Wastewater resource recovery via the Enhanced Biological Phosphorus Removal and Recovery (EBP2R) process coupled with green microalgae cultivation

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DTU Environment
Department of Environmental Engineering

PhD Thesis
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Preface

This thesis is based on the work carried out at the Department of Environmental Engineering at the Technical University of Denmark from October 2012 to October 2015. This thesis was prepared as part of the Integrated Water Technology (InWaTech) project between the Technical University of Denmark and the Korean Advanced Institute of Science and Technology (DTU-KAIST, http://www.inwatech.org.www6.sitecore.dtu.dk/). The research was performed under the main supervision of Associate Professor Benedek Gy. Plósz (DTU Environment) and co-supervision of Professor Barth F. Smets (DTU Environment).

The thesis is organized in two parts: the first part puts into context the findings of the PhD in an introductive review; the second part consists of the papers listed below. These will be referred to in the text by their paper number written with the Roman numerals **I-V**.

I **Valverde-Pérez, B.**, Ramin, E., Smets, B.F., and Plósz, B. Gy., 2015. EBP2R – An innovative enhanced biological nutrient recovery activated sludge system to produce growth medium for green microalgae cultivation. *Water Research*, **68**, 821-830.

II **Valverde-Pérez, B.**, Fuentes-Martínez, J.M., Flores-Alsina, X., Gernaey, K.V., Huusom, J.K., Plósz, B. Gy., 2015. Control structure design for resource recovery using the enhanced biological phosphorus removal and recovery (EBP2R) activated sludge process combined with green microalgae cultivation. *Submitted manuscript*.

III **Valverde-Pérez, B.**, Wágner, D.S., Gülay, A., Smets, B.F., Plósz, B. Gy., 2015. Start-up of the low-sludge age EBPR process – microbial and biochemical process characterization. *Manuscript in preparation*.

IV Wágner, D.S., **Valverde-Pérez, B.**, Sæbø, M., Bregua de la Sotilla, M., Van Wagenen, J., Smets, B.F., Plósz, B. Gy., 2015. Towards a consensus green microalgal growth model (ASM-A) – uptake and storage of nutrients. *Submitted manuscript*.

V Fang, L.L., **Valverde-Pérez, B.**, Damgaard, A., Plósz, B. Gy., Ryg-aard, M., 2015. Life cycle assessment as development and decision support tool for wastewater resource recovery technology. *Water Research, In press*.

In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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In addition, the following authored or co-authored publications, not included in this thesis, were also concluded during this PhD study:

- **Valverde-Pérez, B.**, Wágner, D. S., Kim, G.Y., Han, J.I., Smets, B.F., Plósz, B. Gy., 2015. Extension of the green microalgal growth model ASM-A to predict lipid accumulation in mixed cultures. *In preparation*.
- Wágner, D.S., Radovici, M., Angelidaki, I., **Valverde-Pérez, B.,** Plósz, B.Gy., 2015. Bioflocculation of green microalgae with activated sludge and its application in algae harvesting and biogas production. *In preparation*.
- **Valverde-Pérez, B.**, Mauricio-Iglesias, M., Sin, G., 2015. Systematic design of an optimal control system for the SHARON-Anammox process. *Journal of Process Control*, Accepted with minor revision.
- **Valverde-Pérez, B.**, Fuentes-Martínez, J.M., Flores-Alsina, X., Gernaey, K.V., Huusom, J.K., Plósz, B. Gy., 2015. Control Structure Design of an Innovative Enhanced Biological Nutrient Recovery Activated Sludge System Coupled with a Photobioreactor. *Computer - Aided Chemical Engineering,* **37**, 2555–2560.
- Van Wagenen, J., Holdt, S.L., De Francisci, D., **Valverde-Pérez, B.**, Plósz, B.Gy., Angelidaki, I., 2014. Microplate-based method for high-throughput screening of microalgae growth potential. *Bioresource Technology*, **169**, 566-572.
- Vangsgaard, A.K., Mauricio-Iglesias, M., **Valverde-Pérez, B.**, Gernaey, K.V., Sin, G., 2013. pH variation and influence in an autotrophic nitrogen removing biofilm system using an efficient numerical solution strategy. *Water Science and Technology*, **67 (11)**, 2608–2615.

This PhD study also contributed to international conferences with the following proceeding papers:

- **Valverde-Pérez, B.**, Wágner, D.S., Cecchin, F., Jensen, C.J., Smets, B.F., Plósz, B. Gy., 2015. Impact of operational conditions and process configuration on process performance and microbial community in short solid retention time EBPR systems. Poster presentation, 1st IWA Resource Recovery Conference, Ghent, Belgium.

- Wágner, D.S., **Valverde-Pérez, B.**, Sæbø, M., Bregua de la Sotilla, M., Van Wagenen, J., Smets, B.F., Plósz, B. Gy., 2015. Wastewater resource recovery with green microalgae – modeling the microalgal growth, nutrient uptake and storage using ASM-A. Poster presentation, 1st IWA Resource Recovery Conference, Ghent, Belgium.
- Fang, L.L., **Valverde-Pérez, B.**, Damgaard, A., Plósz, B. Gy., Rygaard, M., 2015. Life cycle assessment as decision support tool in early stage development of a new technology for wastewater resource recovery. Oral presentation, 8th Conference of the International Society for Industrial Ecology, University of Surrey, Guildford, UK.
- Wágner, D.S., **Valverde-Pérez, B.**, Sæbø, M., Bregua de la Sotilla, M., Van Wagenen, J., Smets, B.F., Plósz, B. Gy., 2015. Modelling green microalgal growth, nutrient uptake and storage in the ASM framework. Oral presentation, 9th IWA Symposium on Systems Analysis and Integrated Assessment, Gold Coast, Australia.
- **Valverde-Pérez, B.**, Fuentes-Martínez, J.M., Flores-Alsina, X., Wágner, D.S., Gernaey, K.V., Huusom, J.K., Plósz, B. Gy., 2015. Control structure design for an EBP2R process operated as a sequencing batch reactor. Poster presentation, 9th IWA Symposium on Systems Analysis and Integrated Assessment, Gold Coast, Australia.
- Fang, L.L., **Valverde-Pérez, B.**, Damgaard, A., Plósz, B. Gy., Rygaard, M., 2015. Life cycle assessment as decision support tool for development of a resource recovery technology. Poster presentation (*best poster award*), 9th IWA Symposium on Systems Analysis and Integrated Assessment, Gold Coast, Australia.
- **Valverde-Pérez, B.**, Ramin, E., Smets, B.F., Plósz, B. Gy., 2014. An innovative activated sludge system for enhanced resource recovery via downstream cultivation of green microalgae. Poster presentation, IWA World Water Congress and Exhibition, Lisbon, Portugal.
- Wágner, D.S., **Valverde-Pérez, B.**, Sæbø, M., Van Wagenen, J., Angelidaki, I., Smets, B.F., Plósz, B. Gy., 2014. The effect of light on mixed green micro-algal growth: experimental assessment and modelling. Poster presentation, IWA World Water Congress and Exhibition, Lisbon, Portugal.

- Bregua de la Sotilla, M., Wágner, D.S., **Valverde-Pérez, B.**, Van Wagenen, J., Angelidaki, I., Smets, B.F., Plósz, B.Gy., 2014. Modelling and assessment of the storage of nutrients in a mixed green microalgae culture. Oral presentation, 2nd International Conference on Algal Biorefinery, 2014, Kgs. Lyngby, Denmark.
- Wágner, D.S., **Valverde-Pérez, B.**, Sæbø, M., Van Wagenen, J., Angelidaki, I., Smets, B.F., Plósz, B. Gy., 2014. An Activated Sludge Model for Mixed Green Microalgae (ASM-A): model identification and calibration. Oral presentation, YAS2014: Young Algaeneers Symposium 2014, Montpellier-Narbonne, France.
- **Valverde-Pérez, B.**, Wágner, D.S., Sæbø, M., Van Wagenen, J., Angelidaki, I., Smets, B.F., Plósz, B. Gy., 2014. A green micro-algal growth model developed in the activated sludge modeling framework. Poster presentation, 4th IWA/WEF Wastewater Treatment Modelling Seminar, Spa, Belgium.
- **Valverde-Pérez, B.**, Smets, B.F., Plósz, B. Gy., 2013. Innovative two-stage engineering solutions for resource recovery via downstream cultivation of green microalgae. Oral presentation, 9th IWA International Conference on Watereuse, 2013, Windhoek, Namibia.
- Sæbø, M., **Valverde-Pérez, B.**, Van Wagenen, J., Angelidaki, I., Smets, B.F., Plósz, B. Gy., 2013. A mixed green micro-algae model (MAMO) – model identification and calibration using synthetic medium and nutrient rich carbon depleted wastewater. Oral presentation, 86th Annual Water Environment Federation Technical Exhibition and Conference (WEFTEC), Chicago, USA.
- Mauricio-Iglesias, M., **Valverde-Pérez, B.**, Sin, G., 2013. Selection of controlled variables in bioprocesses. Application to a SHARON-Anammox process for autotrophic nitrogen removal. Poster presentation, 9th European Congress of Chemical Engineering, The Hague, Nederlands.
- Van Wagenen, J., De Francisci, D., **Valverde-Pérez, B.**, Holdt, S.L., Podevin, M., Smets, B.F., Plósz, B. Gy., Møller, P., Angelidaki, I., 2013. Microalgae biorefinery – industrial symbiosis. Poster presentation, Copenhagen Bioscience Conference: Cell Factories and Biosustainability, Hillerød, Denmark.

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Summary

Conventionally, the objective of wastewater treatment has been the elimination of organic and inorganic pollutants, such as nitrogen and phosphorus, from wastewater. Current research promotes a paradigm shift, whereby wastewater is considered not only as a source of pollution but also as a source of nutrients, fresh water and renewable energy. This new approach redefines the conventional wastewater treatment plant (WWTP) as a biorefinery from where different streams are split, each of them rich in different resources. Since many wastewater treatment infrastructures were built about 30 years ago, there is an opportunity of including these novel technologies as part of the future retrofitting and enlargements of the plants. Nevertheless, most of the proposed resource recovery strategies suffer from intensive use of chemicals or energy. In extreme cases, the environmental impact of the technology by itself completely counters the benefit of resource recovery.

As an alternative, this thesis proposes a new fully biochemical resource recovery process, referred to as TRENS. The TRENS consists of an enhanced biological phosphorus removal and recovery (EBP2R) process combined with a photobioreactor (PBR). The EBP2R process is operated at relatively low solid retention time (SRT). Hence the bulk of nitrogen is preserved as ammonium, which is the preferred nitrogen source for green micro-algal growth. The effluent criterion for the EBP2R is set to meet the micro-algal nutrient requirements in terms of nitrogen and phosphorus. To this end a phosphorus-rich stream (referred to as P-stream) is diverted from the anaerobic phase of the EBP2R and combined with a nitrogen-rich stream (referred to as N-stream). As a function of the SRT and the P-stream diversion rate, different nitrogen-to-phosphorus ratios (N-to-P ratio) can be produced, thereby meeting the nutrient requirements of different micro-algal species. Organic carbon oxidation is minimized due to the low SRT. Therefore, most of the organic carbon is incorporated to the sludge via microbial assimilation or storage and conveyed to the anaerobic digester for biogas production. The fraction of nitrogen which cannot be recovered is removed via completely autotrophic nitrogen removal (CANR).

First, a feasibility assessment of the EBP2R process as an algal culture media generator was carried out using continuous-flow and sequencing batch reactor (SBR) configurations. Systems were modelled using the activated sludge

model 2d (ASM-2d). Regardless of the process configuration, factors that can potentially limit nutrient recovery comprise the system SRT and the nitrate recirculated to the anaerobic phase/reactor. Additionally, continuous-flow EBP2R systems can suffer from phosphorus starvation in the aerobic reactors as a result of excessive P-stream diversion. Furthermore, in continuous-flow mode, the P-stream diversion increases the aerobic SRT, while the system SRT is kept. Consequently, nitrifying bacteria can proliferate in the continuous system oxidizing ammonia to nitrate. Therefore, at high P-stream flow diversions polyphosphate accumulating organisms (PAOs) may be outcompeted by denitrifying bacteria. The sequencing EBP2R yielded to higher phosphorus recovery than the continuous flow system. For each of the EBP2R configurations a control structure has been developed and tested using a set of dynamic influent disturbance scenarios. The sequencing EBP2R system was found to be sensitive to large input disturbances. Special care should be taken when tuning the controllers for the sequencing EBP2R to avoid too aggressive control actions that can potentially destabilize the system. Under dynamic conditions, the sequencing EBP2R show better performance in terms of phosphorus recovery and effluent quality (i.e. optimal N-to-P ratio fed to the PBR) than the continuous flow system.

Second, two short SRT EBPR systems were implemented as laboratory-scale continuous-flow and SBR reactor systems. Both systems suffered from extreme filamentous bulking (sludge volume index, SVI>1000 ml/g). Via 16rRNA amplicon sequencing we identified *Thiothrix* as the main filamentous bacteria driving activated sludge settleability. *Thiothrix* proliferated in the reactors when sulphate was reduced to sulphur reduced compounds, such as sulphide, by sulphate reducing bacteria (SRBs). Phosphorus removal was poor during the filamentous bulking event, which was a consequence of the interactions between SRBs and PAOs in the anaerobic phase. SRBs can compete with PAOs for volatile fatty acids under anaerobic conditions. Additionally, sulphide can inhibit phosphorus release by PAOs. As a result, PAOs were washed out from the systems. Filamentous bulking was mitigated and phosphorus removal was restored by reducing the anaerobic SRT of the SBR. However, this strategy failed when applied to the continuous flow system, where only the SVI could be improved.

When extending the aforementioned studies to include the PBR, we identified the lack of a model suitable to describe resource recovery from wastewater

via green micro-algal cultivation. Furthermore, neither of models published in literature were compatible to interface with ASM-2d. Therefore, the third part of the PhD project focusses on the development of a process model for micro-algal growth and substrate storage kinetics (referred to as ASM-A). To facilitate the integration in already well-stablished simulation platforms for wastewater treatment, e.g., the Benchmark Simulation Models 1 and 2, ASM-A was implemented as an extension to the ASM-2d. A set of experiments at different laboratory-scales (microbatch, 1-litre and 24-litre SBR) was designed to generate data for model identification. Furthermore, an independent data set was used for model evaluation. The ASM-A can effectively predict the algal biomass growth, as well as the ammonium and phosphorus concentrations in the bulk liquid and the microbial stored phosphorus. Conversely, our results suggest that the maximum uptake rate parameter for nitrate can be significantly affected by culture history. Therefore the prediction of bulk nitrate concentration and the microbial stored nitrogen requires case-specific model calibration.

Finally, the models developed in PhD project were used to provide data for the inventory of a life cycle assessment (LCA) of the TRENS system implemented in the Copenhagen area. The LCA highlighted the benefits of recovering nutrients but also suggested that heavy metals can potentially impose a bottleneck when reusing water and nutrients from the used water.

Overall, this thesis describes the early stage design of the TRENS system, where model-based studies and laboratory-scale experiments have been used to define the optimal process operation and address future research needs.

Dansk sammenfatning

Historisk og konventionelt set har formålet med spildevandsrensning været at fjerne organiske og uorganiske næringsstoffer, såsom kvælstof og fosfor, fra spildevandet. Denne tilgang er langsomt ved at ændres gennem et paradigmeskifte drevet af aktuel forskning. I det nye paradigme betragtes spildevandet ikke kun som en kilde til forurening, men også som en kilde til næringsstoffer, ferskvand og vedvarende energi. Denne nye tilgang redefinerer det konventionelle rensningsanlæg som værende et bioraffinaderi, hvorfra flere forskellige ressourcerige strømme kan opdeles og udtrækkes. Eftersom mange rensningsanlæg blev bygget for mere end 30 år siden, er der mulighed for at inkludere disse nye teknologier som en del af den fremtidige modernisering og udvidelse af rensningsanlæggene. En stor del af de foreslåede ressourcegenindvindingsstrategier forudsætter dog intensiv anvendelse af kemikalier eller energi. I ekstreme tilfælde overstiger den miljømæssige påvirkning ved anvendelse af teknologien den miljømæssige gevinst forbundet med ressourcegenindvindingen.

Som et alternativ foreslår denne afhandling en ny fuldt ud biokemisk ressourcegenindvindingsproces kaldet TRENS. TRENS består af en forbedret biologisk fosforfjernelse og genindvindingsproces (EBP2R: enhanced biological phosphorus removal and recovery) kombineret med en fotobioreactor. EBP2R processen styres ved en relativt lav slamalder, hvorved størstedelen af kvælstof er bibeholdt som ammonium, hvilket er den foretrukne kilde til kvælstof i forhold til dyrkning af grønne mikroalger. Udledningskriteriet for EBP2R processen er at opfylde mikroalgernes næringsstofbehov i form af fosfor og kvælstof. Med henblik herpå afledes en fosforrig strøm (benævnt P-strømmen) fra den anaerobe fase af EBP2R processen, hvilket kombineres med en kvælstofrig strøm (benævnt N-strømmen). Som en funktion af slamalderen og afledningsraten af P-strømmen kan forskellige kvælstof-fosfor ratioer (N-til-P ratio) produceres, hvorved næringsstofbehovet for forskellige mikroalgearter kan imødekommes. Oxidering af organisk kulstof er minimeret på grund af den lave slamalder anvendt i processen. Dette bevirker, at størstedelen af det organiske kulstof er inkorporeret i slammet gennem mikrobiel assimilation eller opbevaring, og kulstoffet transporteres derefter til den anaerobe rådnetank for produktion af biogas. Fraktionen af kvælstof som ikke kan genindvindes fjernes via fuldstændig autotrof kvælstoffjernelse (CANR: completely autotrophic nitrogen removal).

Først gennemførtes en forundersøgelse af EBP2R processen som en kilde til algedyrkningsmedie. Dette foregik ved anvendelse af kontinuerligt flow og sekventiel batch reaktor (SBR) konfigurationer, hvilke blev modelleret ved hjælp af Activated Sludge Model-2d (ASM-2d). Uafhængigt af reaktorkonfigurationen kan slamalderen i systemet samt nitraten, der recirkuleres til den anaerobe fase/reaktor, potentielt begrænse genindvindingen af næringsstoffer. Derudover kan EBP2R systemer med kontinuerligt flow lide af fosformangel i de aerobe reaktorer som følge af for høj afledning af P-strømmen. Ved kontinuerligt flow medfører afledningen af P-strømmen en stigning i den aerobe slamalder, mens systemets overordnede slamalder bibeholdes. Resultatet af dette er, at nitrificerende bakterier kan profilere i det kontinuerte system og derved oxidere ammoniak til nitrat. Som følge heraf kan polyfosfat-akkumulerende bakterier (PAO) blive udkonkurreret af denitrificerende bakterier ved høj afledning af P-strømmen. Det sekventielle EBP2R system medførte højere fosforgenindvinding end det kontinuerlige flow system. For begge EBP2R konfigurationer blev en kontrolstruktur udviklet og testet ved at anvende dynamisk inputforstyrrelsesscenarier. Det sekventielle EBP2R system viste sig at være følsom over for store inputforstyrrelser. Det er derfor vigtigt, at der udvises særlig omhu, når kontrolsystemet for det sekventielle EBP2R system justeres, således at man undgår for aggressive kontrolaktioner, der potentielt kan destabilisere systemet. Under dynamiske betingelser viser det sekventielle EBP2R system bedre resultater end det kontinuerlige flow system med hensyn til fosforgenindvinding og udløbskvalitet (optimal N-til-P ratio forsynet til fotobioreaktoren).

Dernæst blev to EBPR systemer med lav slamalder implementeret i laboratorieskala som hhv. kontinuerligt flow og SBR reaktor systemer. Begge systemer led af trådformet bulking (slamvolumenindex, SVI>100 mL/g). Ved anvendelse af 16s rRNA amplicon sekventering identificerede vi Thiothrix som værende den dominerende trådformet bakterie, som påvirkede det aktive slams sedimentationsevne. Thiothrix prolifererede i reaktorerne, når sulfat blev reduceret til reducerede svovlforbindelser, såsom sulfid, af sulfatreducerende bakterier (SRB). Fosforfjernelse var ringe, når der var trådformet bulking, hvilket var en konsekvens af interaktionerne mellem SRB'er og PAO'er i den anaerobe fase. SRB'er kan konkurrere med PAO'er for flygtige fedtsyrer under anaerobe forhold. Derudover kan sulfid inhibere fosforfrigivelsen fra PAO'er. Som følge heraf blev PAO'erne udvasket fra systemerne. Trådformet bulking blev nedsat og fosforfjernelsen blev genoprettet ved at redu-

cere den anaerobe slamalder i SBR'en. For det kontinuerlige flow system kunne kun SVI'en forbedres ved anvendelse af denne strategi.

Når de førnævnte undersøgelser udvides til også at inkludere fotobioreaktoren, identificerede vi en mangel på en velegnet model til at beskrive ressourcegenindvindingen fra spildevand via dyrkning af grønne mikroalger. Derudover havde ingen af de i litteraturen beskrevne modeller en berøringsflade kompatibel med ASM-2d. Derfor fokuserer den tredje del af afhandlingen på udviklingen af en procesmodel til at beskrive væksten af mikroalger samt substratlagringskinetik (benævnt ASM-A). For at facilitere integrationen i veletablerede simuleringsplatforme for spildevandsrensning, fx Benchmark Simulation Model 1 og 2, blev ASM-A implementeret som en udvidelse af ASM-2d. Et sæt af eksperimenter på forskellige laboratorieskalaer (mikrobatch, 1 liter og 24 liter SBR) blev designet for at genere data til at identificere modelparametre. Derudover blev et uafhængigt datasæt anvendt til at evaluere modellen. ASM-A kan effektivt forudsige væksten i algebiomasse, koncentrationen af ammonium og fosfor i vandfasen samt det mikrobielt lagret fosfor. Resultaterne indikerer også, at parameteren for den maksimale optagelserate for nitrat kan blive signifikant påvirket af bakteriekulturens historie. Som følge heraf kræver forudsigelsen af nitratkoncentrationen i vandfasen og det mikrobielt oplagrede kvælstof case-specifikke modelkalibreringer.

Endeligt blev de i afhandlingen udviklede modeller anvendt til at levere data til en livcyklusvurdering af TRENS systemet implementeret i Københavnsområdet. Livcyklusvurderingen fremhævede fordelene ved at genindvinde næringsstofferne, men indikerede også, at tungemetaller potentielt kan udgøre en flaskehals, når vand og næringsstoffer genanvendes fra spildevandet.

Samlet set har denne afhandling beskrevet den tidlige designfase for TRENS systemet, hvor modelbaserede studier og eksperimenter i laboratorieskala er benyttet til at definere den optimale drift og adressere fremtidige forskningsbehov.

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Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
BSM1	Benchmark simulation model 1
CANR	Completely autotrophic nitrogen removal
CAS	Conventional activated sludge
CFS	Continuous flow reactor
COD	Chemical oxygen demand
CV	Controlled variable
DO	Dissolved oxygen
EBPR	Enhanced biological phosphorus removal
EBP2R	Enhanced biological phosphorus removal and recovery
ED	Entner Doudoroff
EM	Embden Meyerhof
GAO	Glycogen accumulating organism
GHG	Greenhouse gas
GSA	Global sensitivity analysis
HRT	Hydraulic retention time
IMC	Internal model control
LCA	Life cycle assessment
LCI	Life cycle inventory
N-to-P	Nitrogen to phosphorus molar ratio
PAO	Polyphosphate accumulating organisms
PBR	Photbioreactor
PHA	Polyhydroxialcanoates
qFISH	Quantitative fluorescence in situ hybridization
Q_P	P-stream flow rate
Q_W	Wastage flow rate
RAS	Recirculation of activated sludge
SBR	Sequencing batch reactor
SRB	Sulphate reducing bacteria
SRT	Solids retention time
SVI	Sludge volume index
TAG	Triacylglycerol
TCA	Tricarboxylic acid

TN	Total nitrogen
TP	Total phosphorus
VFA	Volatile fatty acids
WRRF	Water resource recovery facilities
WWTP	Wastewater treatment plant

1 Water resource recovery facilities

The activated sludge process was discovered more than 100 years ago (Schneider 2014). Originally, the conventional activated sludge (CAS) process was designed for organic carbon and nitrogen removal from sewage through bacteria cultivation. Nowadays, the CAS process is a mature technology used worldwide for wastewater treatment prior to discharge into the receiving water bodies. This process reduces the anthropogenic environmental footprint on the aquatic environment. However, as a consequence of increasing global population, climate change and industrialization, the planet resources are being depleted at an alarming rate (e.g. in the past 50 years fertilizer production has increased by 500% due to agricultural intensification, Foley et al., 2011). Therefore, there is an increasing interest in resource recovery from wastewater, referred to as used water (Verstraete et al., 2009), rather than pollutant destruction through mineralization. To this end, the CAS process should be removed from urban water systems and new water resource recovery facilities (WRRF) should be implemented instead. This new approach considers the conventional wastewater treatment plant (WWTP) as a biorefinery where different streams are split, each of them rich in different resources, such as nutrients, energy, bio-polymers or freshwater (Sheik et al., 2014). Since many of the existing WWTPs were built 20-30 years ago, future system retrofits or enlargements give an opportunity to implement the biorefinery concept (Scherson and Criddle, 2014). In the following subchapters the different alternatives for resource recovery, both well-stablished and emerging technologies, are briefly introduced.

1.1 Energy recovery

Wastewater treatment requires large amounts of energy destined to aeration to support organic carbon and ammonia removal by mineralization to carbon dioxide and nitrogen gas, respectively (Jetten et al., 1997). Traditionally, anaerobic digestion of the produced sludge has been used to produce methane, which can be used to produce heat and power. Therefore, heat can be used to warm different units at treatment plants (e.g. anaerobic digester), while electricity can partially cover the aeration energy demand (Foley et al., 2010). In order to reduce the energy demand from aeration, sewage can also be anaerobically digested (Shoener et al., 2014; Batstone et al., 2015). In this way, the influent chemical oxygen demand (COD) can be directly recovered to methane at a lower energetic cost. The efficiency of treating low strength municipal wastewater in this manner has already been demonstrated (Kim et

al. 2011) and has been proposed as a good short term solution to reduce the energy demand from WWTPs (Batstone et al., 2015). However, energy recovery can be compromised, as up to 40% of the produced methane can be lost dissolved in the effluent (Batstone and Virdis 2014). Furthermore, dissolved methane may be released to the atmosphere, thereby contributing to greenhouse gas (GHG) emissions of WWTPs. Alternatively, the A-stage process pioneered in the 1970s is currently gaining interest (Boehnke, 1978). This system promotes the up-concentration of the influent organic carbon into sludge by microbial assimilation and flocculation at short solid retention times (SRTs) and hydraulic retention times (HRTs), thereby minimizing the COD mineralized to CO2 (Jimenez et al., 2015). A similar system has been recently developed at Queensland University, where an enhanced biological phosphorus removal (EBPR) system has been operated at short SRTs (down to 2 days). Methane production thus is enhanced while phosphorus is biologically removed from the effluent (Ge et al., 2013 and 2015). Furthermore, the growth of polyphosphate accumulating organisms (PAOs), which are involved in the phosphorus removal process, promotes the accumulation of COD as polyhydroxialcanoates (PHAs) in the biomass. The digestion of PHA stored in the biomass can further enhance methane production from anaerobic digestion (Wágner et al., 2015a). Generally, short SRT systems produce more easily degradable sludge than conventional secondary sludge, which usually requires pretreatment in order to improve hydrolysis rates and methane yields (Kuglarz et al., 2013). Nevertheless, short SRT processes still pose some challenges due to inefficient COD removal derived from, as example, poor flocculation of the colloidal COD (Meerburg et al., 2015). Further research is still needed before short SRT systems can be implemented at large scale. Despite anaerobic digestion is a technology widely used for methane production, the low economical value of methane has pushed research towards the production of other valuable liquid fuels (Agler et al., 2010). An example of this novel approach is the production of acetate, an anaerobic digestion intermediate, which can be used to produce electricity using microbial fuel cells (Shoener et al., 2014) or to produce lipids (Vajpeyi and Chandran, 2015). Nevertheless, many challenges exist, being separation of anaerobic digestion intermediates the most important. Further research is needed before these processes could be up-scaled (Batstone and Virdis, 2014).

In addition to biogas production from anaerobic digestion, there is growing interest in producing biodiesel by transesterification from lipid accumulating organisms. For example, there is potential to produce biodiesel from filamen-

tous organisms (e.g. *Microthrix parvicella*) able to accumulate lipids as triacylglycerols (TAG) and free fatty acids (Sheilk et al., 2014). However, phototrophic microorganisms, and more specifically green microalgae, are the most studied alternative for biodiesel production (Mata et al., 2010). Phototrophic processes have the potential to produce 280-400% of the anaerobic energy per m^3 of treated sewage (Shoeren et al., 2014). On the other hand, it is known that the biodiesel potential of green microalgae is largely limited by the harvesting, extraction and lipid transesterification costs (Mata et al., 2010). It has been demonstrated that when the lipid content in algae is lower than 40% of dried weight it is more cost-effective to directly convert the wet biomass to biogas through digestion (Sialve et al., 2009) or to other liquid biofuels through hydrothermal liquefaction (López-Barreiro et al., 2014) rather than biodiesel production. Furthermore, it is crucial to use the nutrients and the water content of the used water to make the process sustainable. Otherwise the GHG emissions associated with fertilizer production and the water demand could overwhelm the environmental impact of algal based technologies (Clarens et al., 2009).

1.2 Nutrient recovery

Crop production needs to double in order to supply world population growth, dietary changes and increasing bioenergy use (Foley et al., 2011). As a consequence, the fertilizer industry will need to expand to meet new agricultural requirements. Fertilizers are mainly made of phosphorus, nitrogen and potassium. While potassium supply is not considered a major concern due to its availability (there are available reserves for the next 300 years given the present consumption rate, Batstone et al., 2015), the situation is dramatically different for nitrogen and phosphorus. Phosphorus is mainly obtained from phosphorus rock, a non-renewable source which is expected to be depleted in the next 50-100 years (Cordell et al., 2009). Nitrogen is available as a non-reactive compound in the atmosphere. However, nitrogen fixation through the Haber-Bosch process requires about 2% of global energy consumption, making energy savings the main driving force for nitrogen recovery (Matassa et al., 2015). Nutrient management could improve through better agricultural practices (e.g. minimizing nutrient leaching from fertilizers) or modifying prevailing diets in society (e.g. inefficient protein incorporation in meat). Nevertheless, large amounts of nutrients are excreted and conveyed into WWTPs, which offers a great opportunity for recycling (Cordell et al., 2009). Furthermore, nutrient removal processes are energy intensive and can contribute to GHG emissions from the treatment plant. For example, nitrogen

removal through conventional nitrification-denitrification requires about 40MJ/kg-N removed, the same energy required per kg-N fixated. These processes contribute to GHG emissions from N_2O emissions (Matassa et al., 2015).

Traditionally, nutrients have been recycled through sludge disposal on land as natural fertilizer for crops (Fytili and Zabaniotou, 2008). Studies demonstrate that activated sludge, either stabilized or untreated, contains more available phosphorus for plant uptake than conventional mineral fertilizers (Kahiluoto et al., 2015). This is especially relevant for activated sludge generated at EBPR based treatment plants, which has relatively high phosphorus content (Yuan et al., 2012). However, it is important to stabilize the sludge prior application to land to avoid GHG emissions in the form of N_2O or CH_4 (Yoshida et al., 2015), even though the stabilization process severely reduces the phosphorus mobility and thus hinders plant uptake (Kahiluoto et al., 2015). Moreover, sludge use on land is limited by the heavy metal or emerging micropollutants content in the sludge. Heavy metals and xenobiotics can accumulate in the plant tissue, thereby being introduced into the food-chain (Hospido et al., 2010). As an alternative to sludge application on land, European countries are increasingly using sludge incineration (Kalessidis and Stasinakis, 2012). The sludge ashes have a phosphorus content ranging from 10 to 25% and usually have to undergo a sequence of physicochemical processes for phosphate extraction, which is free of heavy metals (Donatello and Cheeseman, 2013).

Nutrients can also be recovered via chemical precipitation, with struvite precipitation being the most common approach (de Bashan and Bashan, 2004). Struvite is a precipitate formed by magnesium, ammonium and phosphate in a 1-to-1 molar basis (Le Corre et al., 2009). Struvite has been perceived more like a problem than a valuable product due to its spontaneous precipitation in specific parts of the WWTP, e.g., in the anaerobic digester. However, struvite is also considered as an efficient slow leaching fertilizer. Other alternatives are the precipitation of phosphate with iron or calcium. While precipitation by supplying iron is widely used for chemical phosphorus removal, it is considered a poor fertilizer due to its low phosphorus mobility (Wilfert et al., 2015). Calcium phosphates are also known to be poor fertilizers characterized by low phosphorus mobility (Bauer et al., 2007). However, since the main component of phosphorus rock is calcium phosphate, it is believed that crystallization via calcium phosphate formation has the highest potential, as most of the phosphorus industry is prepared to treat this material (Morse et al.,

1998). While these precipitates have the same phosphorus content as standard fertilizers, they have to be supplied with additional mineral nitrogen in order to compete with them in the market (Yuan et al., 2012). The use of these approaches may leave streams with high ammonia content, which can either be removed through anaerobic ammonia oxidation (Anammox) or recovered through physicochemical processes, such as ammonia stripping followed by acid wash (Verstraete et al., 2009). A general limitation of these technologies is the need to up-concentrate ortho phosphate in the used water to get a more efficient precipitation process. This is usually achieved by the combination of chemical precipitation with EBPR systems (Yuan et al., 2012). A drawback in common with sludge based fertilization is that chemical precipitation may induce co-precipitation of heavy metals, which may be harmful for the receiving environment (Ma and Rouff, 2012).

Nutrient recovery by assimilation is a novel option still in the research phase (Matassa et al., 2015). The most investigated approach is the use of green microalgae as natural fertilizer (Mulbry et al., 2005). Microalgae are able to uptake and store large amounts of polyphosphate through luxury uptake (Powell et al., 2008). Furthermore, microalgae can be used to produce valuable amino-acid based fertilizers (Romero-García et al., 2012). Some microalgae species, such as *Scenedesmus* or *Chlorella*, have also shown antimicrobial activity which can be used to control pathogen proliferation (Ördög et al. 2004). Despite these potentials, the studies focusing on nutrient recovery through microalgae are very scarce, as the main research areas have been biofuel or high value chemicals production. Further research in algal-based fertilizers applications is still needed (Shilton et al., 2012). Alternatively, algae have been proposed as direct protein supply for humans or animals by incorporating them as food additives (Becker 2007). However, the inherent dangers of the urban and many industrial used waters (i.e. heavy metals, micropollutants and pathogens) pose a risk for human health, thereby making this option less attractive for nutrient recovery. The main disadvantage of these strategies is that there is poor control on the incoming nutrient balance to the PBR, leading to inefficient nutrient removal. Therefore, further treatment may be required prior to discharge to the water bodies or possible water reuse. Other microorganisms used for nitrogen or phosphorus assimilation gaining interest are purple phototrophic bacteria, methanotrophic bacteria or hydrogen-oxidizing bacteria (Hülsen et al., 2014; Bodelier and Steenbergh 2014; Matassa et al. 2015b).

1.3 Water reuse

Water scarcity is a recognized problem for countries from Southern Europe, like Spain or Italy (Bixio et al., 2006). Recent studies have shown, however, that countries with a relatively low water stress index may suffer from severe water scarcity at the regional level (Hybel et al., 2015). Therefore, water reuse should not only be contemplated at the national level but also at the regional level when assessing water supply alternatives (Godskesen et al., 2013).

Water reuse strategies can be grouped in 4 categories: agriculture, industry, urban and recreational and environmental uses. Each of these categories has its own quality standards for water reuse (Bixio et al., 2006). Although there is an on-going effort to identify appropriate policies and encourage water reuse (EC, 2012), the quality standards are set by each country, making difficult to define a universal approach for water reuse. However, since up to 70% of used fresh water is destined to agriculture (Cordell et al., 2009), irrigation with used water is the most common reuse option (Bixio et al., 2006). The quality demanded depends on the type of irrigated crop, where vegetables that are eaten raw have very strict requirements and vegetables that are eaten after processing or non-edible crops can be irrigated with secondary effluents (Bixio and Wintegns, 2006). The main factors to consider when irrigating with recovered water are nutrients, salinity, pathogenicity and heavy metal content (Norton-Brandão et al., 2013). However, recent research proposes that micropollutants should also be taken into account, as they can be assimilated by plants and incorporated into the food chain or lead to antibiotic resistance (Polesel et al., 2015; Michael et al., 2013).

1.4 Other resources

Traditionally, the main emphasis of resource recovery has been placed on energy production, nutrient recovery and water reuse. However, there is a growing interest in atypical resources, like bioplastics, metals or salts.

PHA is a polyester of hydroxialkanoates that is stored as granules by many organisms. Bacteria use it as a carbon or energy storage pool. The most common strategies to enrich biomass in PHA accumulating bacteria are to expose the biomass to feast-famine conditions and to grow PAOs/GAOs (glycogen accumulating organisms) in EBPR systems (Salehizadeh and Van Loosdrecht, 2004). The lower footprint of PHA-based plastics makes them an attractive alternative to petroleum based plastics. Bioplastics can be used to

produce polymeric based materials, such as plastic packages or even medical implant materials, to produce biofuels or as a source of monomers for synthesis of antibiotics, vitamins, aromatics and pheromones in the chemical industry (Chen, 2009). Despite the wide applicability of this material, there are still limitations related to substrate costs, production rates and product quality, which need to be addressed before implementation in full scale (Bluemink et al., 2015).

Metals can be recovered by physicochemical methods requiring chemicals or energy intensive processes, such as hydrometallurgical processes. In addition, these methods may become inefficient for low strength wastewaters. As an alternative, microorganisms can induce the precipitation of some metals, thereby making them available for further reuse. Sulphate reducing bacteria (SRBs) are a good example, as they induce metal precipitation by producing sulphide. This strategy has been used to recover zinc or palladium (Marck et al., 2004; De Corte et al., 2012). Salts are the other important inorganic resource. They are usually recovered through a combination of different techniques, including nanofiltration, reverse osmosis, electrodialysis or chemical processing (Kim, 2011). They are often recovered from high strength streams, thereby facilitating water reuse.

1.5 TRENS system

Several alternatives exist to recover resources from used water. They range from very well established technologies, like anaerobic digestion, to more advanced treatments that are still under development, like nutrient recovery through purple bacteria. Although many alternatives consist of biological processes, most of the proposed resource recovery schemes propose the use of physicochemical processes (Verstraete and Vlaeminck, 2011). The use of chemicals or energy intensive processes for resource recovery may overwhelm the benefits of recovering the different resources (Corominas et al., 2013).

This thesis presents a new, completely biochemical resource recovery process as an alternative. This novel technology, referred to as TRENS (Fig. 1), consists of a two-stage bacterial-algal system, combined with an anaerobic digester and a completely autotrophic nitrogen removal (CANR) system.

The bacteria based reactor is an enhanced biological phosphorus removal and recovery (EBP2R) system (**paper I**). The EBP2R system is defined as a sequence of anaerobic-aerobic reactors and is able to produce an optimal culti-

vation medium for downstream algal cultivation. Biomass is recycled through anaerobic and aerobic reactors, thereby promoting the growth of PAOs (Mino et al., 1998). The EBP2R is operated at relatively short SRT pursuing a dual goal. On the one hand, at low SRT the carbon oxidation can be minimized (Ge et al., 2013), while most of the incoming COD can be recovered via bio-flocculation and microbial assimilation and storage (PHA and glycogen), and conveyed to anaerobic digestion (C-stream on Fig. 1). On the other hand, by keeping low SRT, nitrifying bacteria are washed out of the reactors, thereby reducing the energy demand by aeration and keeping ammonia as the main nitrogen species in the effluent. This is beneficial for the algae, as they usually prefer ammonia over nitrate or nitrite (Cai et al., 2013). The EBP2R process aims to separate and recover resources by diverting a phosphorus rich stream, referred to as P-stream, via a solid-liquid separation unit operation installed downstream the anaerobic reactors. The remaining flow undergoes aerobic treatment, whereby the non-diverted phosphorus is taken up by PAOs, leaving this stream rich in nitrogen (also referred to as N-stream). The effluent nutrient content set for the EBP2R system is controlled by manipulating the flows of P-and N-streams, thereby setting the nitrogen to phosphorus ratio (N-to-P ratio) in the influent to the photobioreactor (PBR). To match the nutrient requirements of the cultivated micro-algal culture, the effluent N-to-P ratio may range from 8 (Christensons and Sims, 2011) up to 100 (Geider and La Roche, 2002) in a molar basis. The main objectives of this approach are to maintain the targeted culture as dominant and to keep the inorganic nutrient content of the PBR effluent to a minimum.

Algal systems can be designed as an open pond reactors, which require less maintenance, or as a closed PBRs, which offer higher photosynthetic yields and allow more controlled cultivation conditions (Muñoz and Guieysse, 2006). Usually, algal based treatment is used as polishing step (Boelee et al., 2011) or optimally integrated in the treatment plant depending on the nutrient flows (Wang et al., 2010; Van Den Hende et al., 2014). Algal based treatment can also be used the downstream to the anaerobic digestion to treat the rejected water rich in nutrients (Kim et al., 2015; van der Ha et al., 2011). Alternatively, algae can be cultivated with bacteria in granular systems, thereby promoting synergies between both microorganisms. Algae can provide oxygen for aerobic growth of bacteria as well as growth-promoting factors, while bacteria produce carbon dioxide through COD mineralization (Muñoz and Guieysse, 2006). Furthermore, the bacterial-algal aggregates can be harvested more effectively by gravity settling (van den Hende, 2014). As discussed ear-

lier in this chapter, these approaches may lead to inefficient nutrient removal, limiting the options for water reuse. The TRENS system overcomes this issue by controlling the N-to-P ratio in the EBP2R effluent, thereby optimizing the growth media and ensuring that nutrients end encapsulated inside the algal biomass. The algal biomass can then be used as natural fertilizer (Mulbry et al., 2005). Furthermore, since in the TRENS system all nutrients are stored inside the algae and the water is low in inorganic nutrients, the algal suspension can be directly applied to land for fertilization and irrigation, thereby avoiding harvesting costs. Finally, carbon dioxide can easily be supplied from the flue gases produced during the biogas combustion in the cogeneration units (Napan et al., 2015).

The layout of the TRENS system comprises anaerobic digestion, which produces methane from biomass, and CANR process, employed to remove a fraction of ammonia which cannot be recovered via micro-algal assimilation. These two technologies are relatively well established, especially the anaerobic digestion process, and are not further studied in this early stage of process development. Further information about these processes may be found elsewhere (Apples et al., 2008; Carballa et al., 2015; Mutlu 2015; Vangsgaard 2013).

1.6 Objectives

The aim of this thesis is to develop the TRENS process, and present the proof-of-concept via laboratory-scale experiments and process modeling. To this end, a wide range of tools have been used, comprising models, different lab-scale reactors and analytical tools (both physicochemical and microbiological), which have been used in both bacterial and algal systems. The weaknesses of some of the tools available in the literature, such as process models, have also been identified and improved through this study. The main goals of the thesis are to:

- Critically evaluate the existing models for EBPR systems and algae considering their usefulness when used to model resource recovery systems in general and, more specifically, the TRENS system (**paper III and IV**).
- Explore the behaviour of EBP2R systems and define the operation window for this system (**paper I**).

- Developing the TRENS process where the chosen algae for cultivation are consortia of *Chlorella sp.* and *Scenedesmus sp.*, additionally considering the system controllability under dynamic conditions to which WWTP are subjected (**paper II**).
- Describe the start-up of short SRT EBPR and define the optimal operation conditions in terms of process stability. To this end, the microbial communities playing important roles should be identified (**paper III**).
- Compare the stability of the short EBPR systems operated using different configurations (**paper II** and **III**).
- Develop a consensus algal model helpful to describe nutrient recovery by green microalgae from wastewater, considering the limitations of existing models (**paper IV**).
- Assess the environmental impacts associated with the TRENS process implemented in Copenhagen suitable to fulfil the water requirement by irrigation in the area, offering an opportunity to re-address the research efforts (**paper V**).

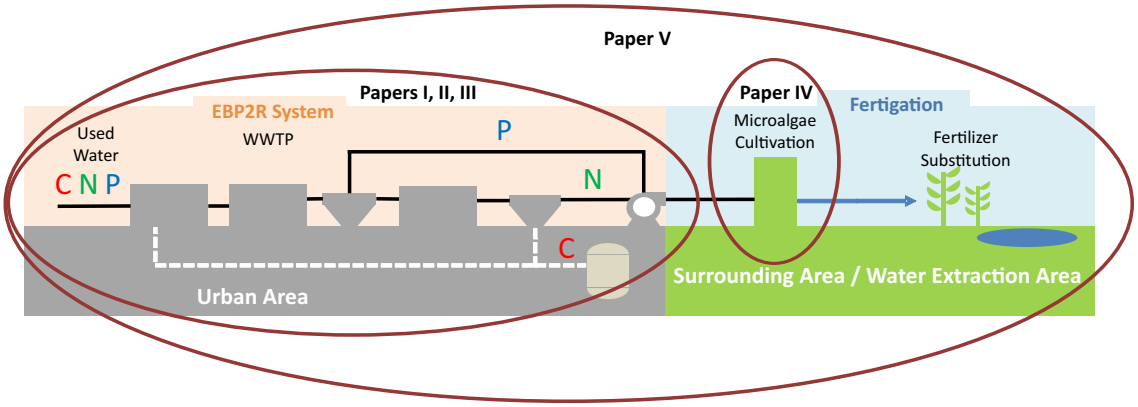

Figure 1. Layout of the TRENS process. It includes an overview of the process unit operations evaluated in each of the journal papers part of this thesis (journal papers are included in the appendix of the thesis).

2 The enhanced biological phosphorus removal and recovery (EBP2R) process

The EBP2R system is the most novel component in the TRENS system and the unit process in charge of manipulating the nutrient balances for optimal algal cultivation. The EBP2R is based on the traditional EBPR systems. Given the increasing concerns about the eutrophication potential of the phosphorus discharged to the aquatic environments, EBPR systems, discovered by the late 1950s, became a biological process used worldwide for phosphorus removal (Henze et al., 2008).

2.1 Microbiology of EBPR systems

EBPR systems rely on the PAOs, which are promoted by recycling the mixed liquor through anaerobic and aerobic conditions (Barnar, 1975). In addition to PAOs, there are a large number of different microbial groups also part of the microbial community of EBPR system (Nielsen et al., 2012). The core microorganisms of EBPR systems are introduced in the following sections.

2.1.1 Polyphosphate accumulating organisms

Polyphosphate accumulating organisms, referred to as PAOs, are those bacteria able to uptake and store large amounts of polyphosphate (up to 12% of their total weight; Nielsen et al., 2012). *Accumulibacter phosphatis* is believed to be a key microbe driving the phosphorus removal as it has been found to be the most abundant PAOs in most of the EBPR plants (abundance ranges from 3 to 22% of total bacteria, Mielczarek et al., 2013; Oehmen et al., 2007). It has been proposed as a model microorganism to describe the biological phosphorus removal mechanism (Seviour et al., 2003).

Based on the *Accumulibacter* biochemical model, illustrated in Fig. 2, the biological removal phosphorus can be explained as follows. Under strict anaerobic conditions (i.e. left side of Fig.2), PAOs can take up VFAs from the media and store them by forming poly-β-hydroxialkanoates (PHAs), being the most common ones poly-β-hydroxybutyrate (synthetized from 2 acetate molecules) and poly-β-hydroxyvalerate (formed from acetate and propionate). Since PAOs cannot ferment the incoming organic carbon to produce volatile fatty acids (VFAs), their activity relies on the presence of VFAs in the influent or the fermentation products from ordinary heterotrophs (Nielsen et al., 2010). VFAs are actively transported across the cell membrane (Mino et al., 1987). The energy needed for VFA uptake is provided by the proton

motive force created by the products of the polyphosphate hydrolysis, namely, phosphate, magnesium and potassium (Saunders et al., 2007). Additionally, the polyphosphate hydrolysis produces ATP by transferring an energy rich phosphoric group from polyphosphate to ADP. The produced ATP is then used for acetate activation to acetyl-CoA (Oehment et al., 2007). Phosphorus release is enhanced at high pH as PAOs need to generate more energy to protonate and take up VFAs (Smolders et al., 1994). Despite the agreement on the fate of VFAs and phosphate under anaerobic conditions, researchers have proposed many different approaches to generate the reducing power need for PHA synthesis. The two widely accepted biochemical models are: i) the Comeau/Wentzel model, which suggests that the reducing equivalents are produced via tricarboxylic acid (TCA) cycle (Comeau et al., 1986; Wentzel et al., 1986); and ii) the model proposed by Mino et al. (1987), which affirms that glycogen is hydrolyzed in order to produce the needed reducing equivalents. Nowadays, there are evidences to believe that PAOs use both mechanisms together to supply enough reducing power to produce PHA (Oehmen et al., 2007). Yet, another disagreement exists in relation to the glycogen degradation pathway: Entner Doudoroff (ED) or Embden Meyerhof (EM), yielding the second one more ATP. While there are strong evidences suggesting that EM is the preferred pathway for glycogen degradation for *Accumulibacter* (García-Martín et al., 2006), the ED cannot be disregarded (Oehmen et al., 2007).

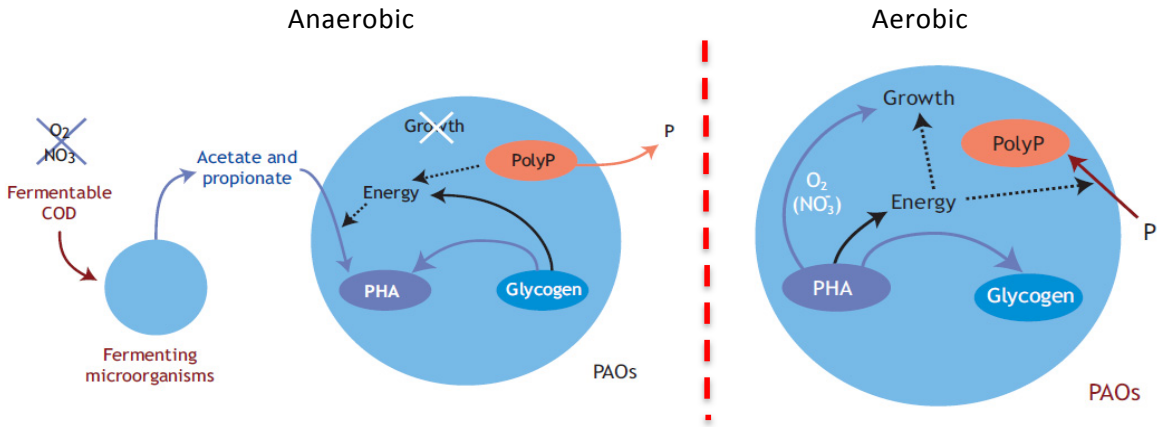

Figure 2. Simplified biochemical model for PAOs under anaerobic (left-side) and aerobic (right-side) conditions (taken from Henze et al., 2008).

Under aerobic conditions the PHA is degraded to acetyl-CoA or propionyl-CoA that further enter in the TCA cycle to produce energy. Both PHA and the produced energy are used for biomass growth. However, part of the gen-

erated ATP is used to take up all the available phosphorus and to replenish glycogen, so PAOs have both energy pools fully supplied and ready to start a new cycle (Oehmen et al., 2007). It should be noted that some clades of *Accumulibacter phosphatis* are able to denitrify, showing a similar behavior as in aerobic environments (i.e. PHA are degraded to sustain microbial growth and replenish the polyphosphate and glycogen, Mielczarek et al., 2013). Right side of Fig. 2 summarizes the aerobic/anoxic metabolic pathway.

Despite the intensive research on the *Accumulibacter* genus, PAOs have been found to be very diverse (Wong et al., 2005; Mielczarek et al., 2013). *Tetrasphaera*, which belongs to the *Actinobacteria* phylum, have been extensively reported as putative PAOs. *Tetrasphaera* are usually more abundant, up to 35% of total bacteria, than *Accumulibacter* in conventional EBPRs (Nielsen et al., 2010). These bacteria are very versatile. Even if all of them can take up and store phosphorus to some extent, only the non-filamentous *Tetrasphaera* are considered to be true PAOs (Nguyen, et al., 2011; Mielczarek et al., 2013). They share many metabolic pathways with *Accumulibacter*, such as the TCA cycle, glycolysis or polyphosphate degradation and synthesis. However, only *T. japonica*, out of the six morphotypes, was able to store PHA. Unlike *Accumulibacter*, *Tetrasphaera* uses the glycogen pool as the only carbon and energy storage supporting growth and active phosphorus uptake in the aerobic phase. In addition, *Tetrasphaera* can also ferment sugars, denitrify or assimilate nitrate to ammonia to support microbial growth (Kristiansen et al., 2013). *Microthrix parvicella*, a filamentous bacteria also belonging to *Actinobacteria*, can accumulate polyphosphate under aerobic conditions if lipids are stored as TAG during the anaerobic phase (McIlroy et al., 2013). Although they have been reported to be less effective than other PAOs, there are cases where *M. parvicella* became the dominant PAO carrying out the biological phosphorus removal (Wang et al., 2014). Other putative PAOs are *Dechloromonas*, belonging to *Rhodocyclales* (Lv et al., 2015), or part of the *Comamonadaceae* family (Ge et al., 2015).

2.1.2 Glycogen accumulating organisms

Glycogen accumulating organisms, referred to as GAOs, are microbes which metabolism is rather similar to PAOs. The main difference is that GAOs cannot take up phosphorus, so they do not contribute to the phosphorus removal. However, GAOs can easily take up VFAs under anaerobic conditions. Therefore, GAOs may outcompete PAOs for VFAs compromising the phosphorus removal (Cech and Hartman, 1993).

In order to take up VFAs, GAOs produce energy through the efflux of protons at expense of ATP (Saunders et al., 2007). Since GAOs do not store polyphosphate, their energy supply depends entirely on glycogen. To produce enough ATP for VFAs uptake the glycogen is degraded to a larger extent than in the case of PAOs, leading to an excess of reducing power. The extra reducing power has to be consumed via pyruvate reduction to propionyl-CoA through the TCA cycle. As a consequence, the stored PHAs by PAOs and GAOs are different even they are fed with the same carbon source (Oehmen et al., 2007). Under aerobic conditions, similarly to PAOs, they oxidize the stored PHAs for biomass growth and energy production and replenish their energy pool, glycogen.

The first GAO characterized belongs to the group of the *Gammaproteobacteria* and was named as *Competibacter* (Nielsen et al., 1999; Crocetti et al., 2002). *Competibacter* competes for acetate with PAOs and may outcompete them at relatively high temperatures (López-Vázquez et al., 2009). More recently, a new group of GAOs, which belongs to the *Alphaproteobacteria*, has been isolated and identified as *Defluvicoccus*-related organisms (Wong et al., 2004; Meyer et al., 2006). *Defluvicoccus*-related GAOs, contrary to *Competibacter*, compete with PAOs for propionate rather than acetate (López-Vázquez et al., 2009). Since GAOs can only utilize their glycogen storage while PAOs can use both, polyphosphate and glycogen, PAOs can tolerate better alkaline environments, in which more energy is required for VFA protonation and uptake (Oehmen et al., 2005). Therefore, GAOs may be outcompeted at pH levels close to 8. GAOs can also be outcompeted by carefully controlling the oxygen level in the aerobic stage, as PAOs show higher affinity than GAOs for oxygen (Carvalheira et al., 2014).

2.1.3 Nitrifying bacteria

Ammonia oxidation to nitrate is a biochemical process carried out by two different microbial guilds: ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB).

AOB are mainly chemilithoautotrophs that use inorganic carbon to build biomass. The energy is derived from the oxidation of ammonia to nitrite in the presence of oxygen. This process, referred to as nitritation, is described by the eq. 1 (Barnes and Bliss, 1983):

$$\begin{gathered} NH_4^+ + 1.382O_2 + 1.982HCO_3^- \rightarrow \\ 0.018C_5H_7NO_2 + 0.982NO_2^- + 1.891H_2CO_3 + 1.036H_2O \end{gathered} \quad (1)$$

All the members of the AOB group belong to the *Proteobateria* phylum. Most of the species are classified as *Gammaproteobacteria* or *Betaproteobacteria*. However, mostly *Betaproteobacteria* AOB are usually found in wastewater systems, mainly *Nitrosomonas* and *Nitrosospira* (Wagner et al., 1996; Nielsen et al., 2010). In EBPR systems, microbial diversity is affected by the ammonia, oxygen and salinity levels (Nielsen et al., 2010). AOB abundance in EBPR systems can go up to 5% (Albertsen et al., 2012).

NOB are also chemolithoautotrophs able to oxidize nitrite to nitrate to produce energy, process referred to as nitratation. NOB also need oxygen to oxidize nitrite. Nitrite oxidation is described as follows (Barnes and Bliss, 1983):

$$\mathrm{NO_2^-} + 0.0025\mathrm{NH_4^+} + 0.4875\mathrm{O_2} + 0.01\mathrm{H_2CO_3} + 0.01\mathrm{HCO_3^-} \rightarrow 0.0025\mathrm{C_5H_7NO_2} + \mathrm{NO_3^-} + 0.0075\mathrm{H_2O} \quad (2)$$

NOB diversity is wider than for AOB, with bacteria from three different phyla: *Proteobacteria*, *Nitrospirae* and *Chloroflexi* (Daims et al., 2006, Sorokin et al., 2012). Despite their wide diversity, only *Nitrobacter sp.* and *Nitrospira sp.* are commonly found in WWTPs. Similar to AOB, the abundance and dominant species depend on the oxygen level, the substrate availability (nitrite in this case) and salinity in EBPR systems (Nielsen et al., 2010). Normal abundance in EBPR systems is around 4% (Albertsen et al., 2012).

2.1.4 Denitrifying bacteria

The term denitrifying bacteria in this thesis applies to those heterotrophic bacteria able to degrade organic carbon to carbon dioxide. To this end, denitrifying bacteria use nitrate or nitrite as alternative electron acceptors - with nitric oxide and nitrous oxides as possible intermediates. Through this process both nitrogen species are reduced to N_2 gas as follows:

$$\mathrm{NO_3^-} + 1.08\mathrm{CH_3OH} + 0.24\mathrm{H_2CO_3} \rightarrow 0.056\mathrm{C_5H_7NO_2} + \mathrm{HCO_3^-} + 1.68\mathrm{H_2O} + 0.47\mathrm{N_2} \quad (3)$$

$$\mathrm{NO_2^-} + 0.67\mathrm{CH_3OH} + 0.53\mathrm{H_2CO_3} \rightarrow 0.04\mathrm{C_5H_7NO_2} + \mathrm{HCO_3^-} + 1.23\mathrm{H_2O} + 0.48\mathrm{N_2} \quad (4)$$

Denitrifiers in EBPR plants rather diverse, primary belonging to *Betaproteobacteria* in the genera *Thauera*, *Azoarcus*, *Zoogloea*, *Curvibacter*, *Accumulibacter*, *Dechloromonas* and *Comamonadaceae* (Nielsen et al., 2010; Osaka et al., 2006). Other *Alphaproteobacteria*, like *Rhodobacteraceae*, or *Gammap-*

roteobacteria, like *Competibacter* or *Pseudomonas*, can also denitrify in EBPR systems. Each species is specialized in a different type of substrate, ranging from organic acids to amino acids or sugars. The wide spectrum of substrates accepted by the denitrifying community suggests that the different bacteria occupy different ecological niches, thereby ensuring functional stability in EBPR systems (Thomsen et al., 2007).

Importantly, denitrification may deteriorate EBPR systems performance. The presence of nitrate or nitrite in non-aerated basins leads to competition between denitrifyers and PAOs for available COD, whereby PAOs are outcompeted as denitrifyers are faster taken VFAs up (Chuang et al., 1996). Since PAOs do not store enough PHA in the anaerobic phase of their growth, PAOs cannot take phosphorus up or use PHA for growth in aerobic environments, leading to their wash-out from the system (Guerrero et al., 2011). Therefore, when EBPR should support both denitrification and phosphorus removal, the system may have to be supplied with external COD.

2.1.5 Filamentous bacteria

Filamentous bacteria are thread-like shaped. Filaments have been traditionally associated with foaming issues in aeration basins and digesters and poor sludge settling properties (Tandoi et al., 2006; Dalmau et al., 2010). They are frequently present in EBPR systems, where its abundance can go up to 25% of total bacteria (Nielsen et al., 2010) – reported using samples taken from Danish WWTPs. The filaments more frequently found in EBPRs are *Microthrix parvicella*, *Chloroflexi*, *Thiothrix* or candidate phylum TM7 (Mielczarek et al., 2012).

Microthrix parvicella is a well-known lipid consumer, able to store long chain fatty acids as TAG under anaerobic conditions (Rossetti et al., 2005). *Microthrix parvicella* is also considered as a putative PAO, able to take up and store phosphates (McIlroy et al., 2013). *Microthrix parvicella* is very versatile, as it can store substrates under anaerobic conditions, it is able to denitrify nitrate to nitrite and effectively grows in aerobic environments. Therefore, the use of selectors may not be helpful to phase out this microbe (Tandoi et al., 2006).

Chloroflexi, despite its relatively high abundance, up to 40% of the total filamentous bacteria, do not significantly contribute to sludge bulking (Wágner et al., 2015b). However, they cover an important role on EBPR systems by hydrolysing complex polymers, so they can be further fermented to VFAs available for other microorganisms. Some have been also reported as denitri-

fying bacteria and NOB (Nielsen et al., 2010; Sorokin et al., 2012). The candidate phylum TM7 is able to grow on a wide variety of organic substrates and also supports protein hydrolysis. However, it is not clear yet whether they can contribute to the fermentation process or not (Nielsen et al., 2009). *Thitothrix* is a filamentous bacteria characterized by the consumption of sulphide under aerobic conditions. It is found in relatively low abundance in EBPR systems treating municipal wastewater, while it may appear at much higher abundances in EBPRs treating industrial wastewaters (Nielsen, et al., 2009). It is worth to mention that some members from the genus *Tetrasphaera* may be also filaments contributing to the filamentous bulking in EBPR systems (Nielsen et al., 2009).

2.1.6 Sulphate reducing bacteria

Sulphate reducers are anaerobic bacteria able to reduce sulphate to sulphur reduced compounds, such as sulphide. Sulphate reducing bacteria (SRBs) can be classified in two different groups depending to what extent they oxidize the organic carbon. Completely oxidizing SRBs are able to take up and oxidize a wide range of molecules to carbon dioxide. However, incomplete oxidizers cannot oxidize beyond acetate. *Desulfobacter* is the most representative bacteria from the first group, while *Desulfovibrio*, *Desulfomicrobium* and *Desulfobulbos* belong to the second one (Hao et al., 2014). SRBs have been reported in WWTPs close to the coastline, where the sulphate content on wastewater is relatively high, promoting their growth (Van den Brand et al., 2015). Importantly, when SRBs were found in lab scale EBPR systems, they were able to both promote and inhibit PAOs (Yamamoto-Ikemoto et al., 1991, 1994, 1996, 1998). So far, no clear competition or inhibition pathways have been stablished between SRBs and PAOs. It is noteworthy to mention that SRBs can effectively promote the growth of *Thiothrix*, thereby leading to filamentous bulking (Yamamoto-Ikemoto et al., 1991).

2.2 Modelling EBPR systems

Two different model families have been applied for EBPR modelling: metabolic models (e.g. Oehmen et al.2010) and Activated Sludge Models (ASM, Henze et al., 2000). Both models consist of sets of stoichiometric and kinetic expressions describing the different biochemical processes in the reactors. In the metabolic models, yield coefficients are estimated based on the theoretical balances over substrate, energy and reducing power - a factor that can reduce the calibration efforts. The ASM families provides a set of default values which are case specific and must be re-estimated for each EBPR. In this

thesis the ASM family models are used to describe the EBP2R, which includes heterotrophs, nitriefiers and PAOs. To be more specific, the ASM-2d, proposed by Henze et al. in 1999 and further extended by Flores-Alsina et al. (2012) by including bacterial decay rates as function of the redox environment, has been used in **papers I, II** and **V**. Additionally, a model for an EBP2R operated as SBR has been built, which was further used in **paper II** for process optimization and control structure design. These models, and any other biological model used in this thesis, were implemented in Matlab-Simulink (The MathWorks, Natick, MA).

2.3 Design and control of the EBP2R system

The EBP2R system is designed according to the empirical guidelines established by Randall et al. (1998) for EBPR systems defined as a sequence of anaerobic-aerobic reactors (i.e. the Phoredox configuration). The design takes into account the influent biological oxygen demand (BOD) as well as the targeted phosphate reduction to assign the anaerobic hydraulic retention time (HRT) and define its relation with the aerobic HRT. The anaerobic volume is divided into two different reactors to enhance the phosphorus release in the anaerobic phase. For the influent wastewater quality, the case of Lundtofte WWTP (Denmark) is chosen, with a content of 712 mg-COD/L of total COD, 54 mg-N/L of total nitrogen (TN) and 9 mg-P/L of total phosphorus (TP) and an inflow rate of 28800m^3/d. The same guidelines have been used to design the continuous flow system (CFS) and the sequencing batch reactor (SBR).

Since the EBP2R is a novel process, the first step is to evaluate the system response to the main parameters expected to drive the process performance in order to get some insights about the process (**paper I**). The parameters considered for process evaluation are the P-stream flow rate (Q_P) and SRT. To this end, a set of scenario simulations were run, where the impact of different combinations of Q_P and SRT on the different key variables (phosphorus recovery, PAOs, nitrifying bacteria, total nitrogen and local SRT) was analysed.

The CFS is analysed in Fig. 3. All profiles in Fig. 3a show an increase in phosphorus recovered along with the Q_P up to a maximum load, which corresponds to the onset of the wash-out of PAOs from the system (Fig. 3b). At high Q_P, phosphorus can still be recovered by the downstream PBR, but without any PAO activity, and therefore without any benefit compared to conventional WWTPs. Fig. 3b and 3c show the concentration of PAOs and autotrophic nitrifiers at three system SRTs as a function of Q_P. PAOs are pro-

gressively washed out when the Q_P increases (Fig.3b). This is because diverting the orthophosphate in the P-stream reduces PAOs growth due to phosphorus starvation in the aerobic reactor. If PAOs do not have phosphorus to take up and store as polyphosphate, they will not able to take up the VFAs once they are back in the anaerobic reactor. Consequently, PAOs grow at lower rates in the aerobic reactor. Phosphate recovery rates increase with the Q_P until the maximum capacity, close to 70% of the influent phosphorus load (Fig. 3a). In other words, as long as PAOs can be retained in the system, releasing the phosphate in the anaerobic reactors, the loss of PAOs activity is over-compensated by the increase of Q_P, increasing the phosphorus load diverted in the P-stream.

The phosphorus recovery can also be limited by nitrifiers (Fig. 3c). Nitrifiers grow in the aerobic reactor despite the low system SRT. This is a consequence of the local SRT in the different reactors (Fig. 3d). Due to the solid-liquid separation process in between the anaerobic and aerobic phases, solids get more concentrated in the aerobic reactor at increasing Q_P. As a result, bacteria spend more time under aerobic conditions at high Q_P, leading to nitrifiers proliferation in the system. Nitrifiers activity can negatively impact the EBP2R performance in two ways. On the one hand, these bacteria produce nitrate, which is recirculated to the anaerobic reactors. In the presence of nitrate denitrifying bacteria compete with PAOs for VFAs. Since PAOs cannot effectively accumulate PHA under anoxic conditions, their growth on stored PHA is limited under aerobic conditions. As a consequence, the PAOs may be washed-out from the system (Fig. 3a and 3c). On the other hand, nitrate rather than ammonia, which is the preferred nitrogen source for most of the algae (Cai et al., 2013), is fed to the PBR. In addition, nitrogen is removed from the media, reducing the nutrient content of the constructed cultivation media (Fig. 3c).

Similar profiles were generated for the SBR. The main difference between the CFS and SBR configurations was the control of the SRT. In the SBR, the wastage of sludge equally affects the anaerobic and the aerobic SRT, as the total biomass is the same in both phases. Consequently, nitrifiers cannot grow at comparably high Q_P. Only at SRTs higher than 5 days nitrifiers could proliferate in the SBR (Fuentes-Martínez et al., 2014).

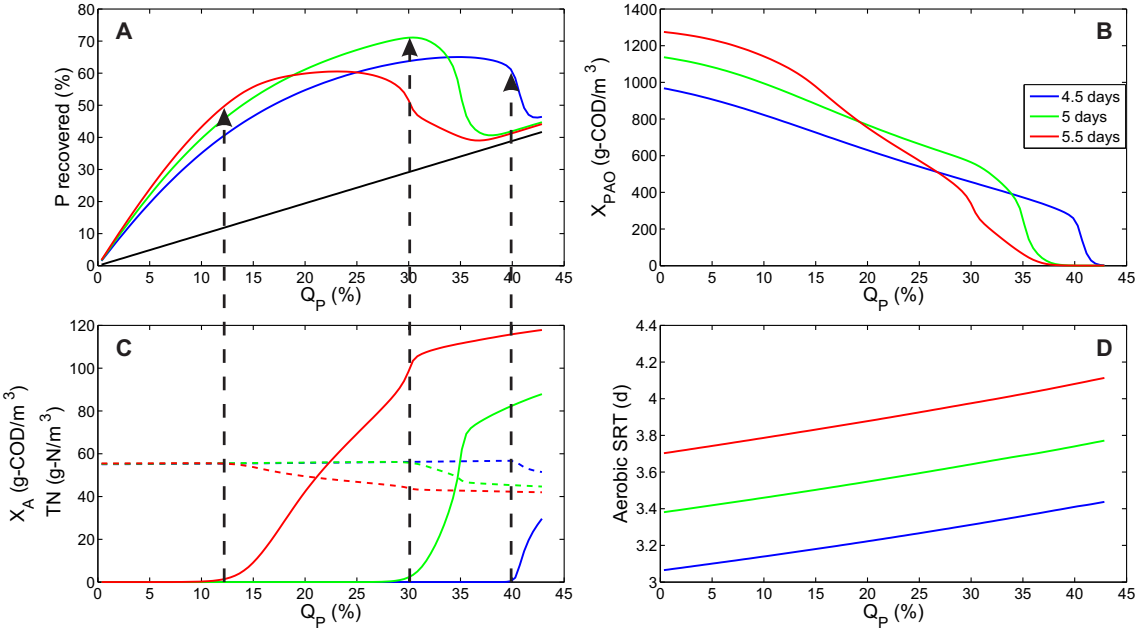

Figure 3. a) Percentage of phosphorus recovered in the P-stream; b) PAOs concentration in the aerobic reactor; c) autotrophs concentration in the aerobic reactor (X_A, continuous line) and total nitrogen in the effluent (TN, dashed line); d) aerobic SRT. Representation at different system SRTs: 4.5 d (blue), 5 d (green) and 5.5 d (red). Arrows relate the onset of effective growth of nitrifiers in the EBP2R with a change of the trend on P-recovery due to the wash-out of PAOs (**Paper I**).

2.3.1 Including process control as part of the process design

Despite being challenging, including process control feasibility assessment at this early stage of process development is considered to be useful to identify process constrains imposed by the control system (Huusom, 2015). Overlooking process control may lead to process configurations or operational conditions where the EBP2R would be impossible to control under dynamic conditions, converting it into a technology unsuitable for full scale application. Therefore, the study in **paper II** aims to apply the findings from **paper I** to design a control system. The control structures were designed following a systematic methodology adapted from literature (Fig. 4).

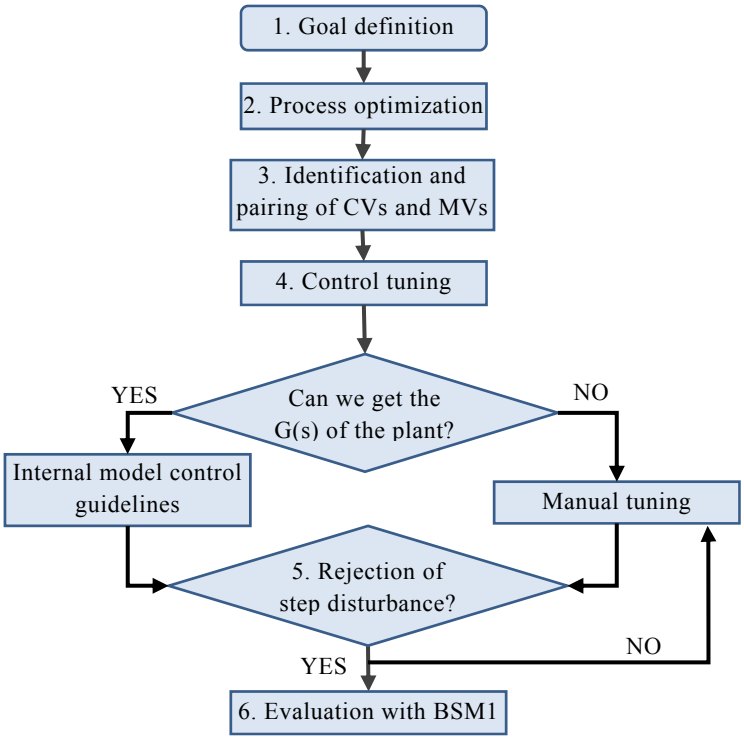

Figure 4. Control structure design methodology (adapted from Valverde-Pérez et al., 2015).

Step 1, the goal definition, is common to both configurations. The overall goal of the TRENS system is to produce an algal suspension rich in nutrients, which will be further used for fertigation. The effluent produced by the TRENS system should be harmless to the environment. To avoid soil pollution and eutrophication of ground water due to infiltration as consequence of fertigation, the nutrients must be encapsulated inside the algae. Therefore, the primary goal of the EBP2R is to keep the N-to-P ratio at the optimal value. For our case study, the N-to-P ratio considered is 17, expressed on a molar basis, which is optimal for the algal consortium used in **paper IV** (Steidl, 2015). If the effluent N-to-P ratio is different, nutrients cannot be taken up and stored by the algae, thereby compromising the effluent quality. The secondary goal of the system is to maximize resource recovery. To this end, phosphorus recovery should be maximized. In case the maximum phosphorus recovery leads to a relatively high N-to-P ratio, part of the nitrogen should be removed via CANR. Conversely, if the maximum phosphorus recovery leads to N-to-P ratios lower than 17, less phosphorus should be recovered via P-stream diversion. Therefore, the maximized nutrient recovery does not compromise the end use in fertigation. The next steps depend on the system configuration (i.e. SBR or CFS).

The process operation for the CFS is optimized and results are shown in Fig. 5. When the SRT is lower than 3.5 days the phosphorus recovery is limited due to the wash out of the PAOs. As a function of the Q_P, operating conditions with SRT higher than 4.5 days may lead nitrifying bacterial growth, thereby oxidizing ammonia to nitrate. The nitrate is recycled to the anaerobic reactors, which promotes denitrification by ordinary heterotrophs over VFA uptake and storage by PAOs. At comparably high Q_P, the growth of PAOs is limited by phosphate in the aerobic reactors, thus leading to their wash out (in agreement with Fig.3a). If the phosphorus recovery is maximized the effluent N-to-P ratio would be 18 (Q_P as 40% of the influent flow rate and SRT of 4.5 days, Fig.5). Therefore, the operation of CFS is limited by phosphorus and the surplus of nitrogen should be removed via CANR.

The selection of the set points should consider the process constrains. Optimal phosphorus recovery can be achieved at SRT=4.5 days and Q_P=40% relative to the influent flow rate, thereby recovering about 70% of the influent phosphorus. However, if the control action requires increasing Q_P to compensate for a phosphorus deficiency it results in further decrease in phosphorus recovery. This is because beyond the 40% diversion the phosphorus recovery decreases at increasing Q_P. As a trade-off between process controllability and maximum phosphorus recovery, the targeted phosphorus recovery is set to 65% of the influent phosphorus. This recovery corresponds to Q_P of 30% at SRT=4.5 days. Finally, enough oxygen should be supplied to sustain heterotrophs and PAOs growth. The chosen set point, based on the scenario simulations carried out in **paper I**, is 1.5 mg/L.

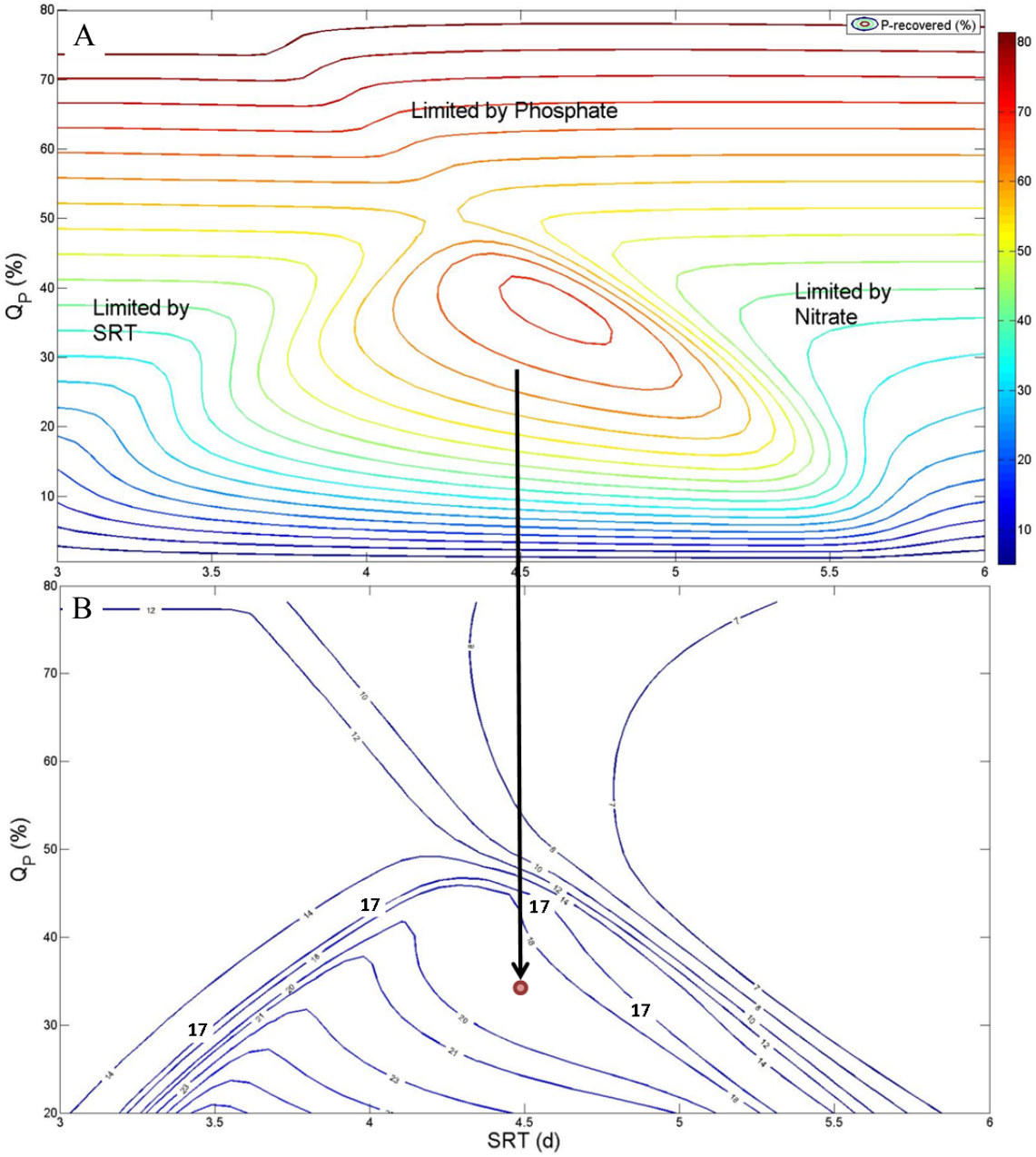

Figure 5. Process performance of the continuous flow EBP2R system: a) Percentage of P recovered from the influent as a function of the P-stream flow and the SRT; b) N-to-P molar ratio in the EBP2R effluent as a function of the P-stream flow and the SRT (**papers I** and **II**).

Results related to the SBR optimization are shown in Fig.6. At SRT<3.5 d, PAOs are washed out from the reactor, thus leading to a poor control on phosphorus recovery. At SRT ranging from 5 to 6 days PAOs activity is limited by nitrification. Nitrifiers oxidize ammonia to nitrate, which becomes available for heterotrophic denitrifiers at the beginning of the anaerobic phase. Similar to the CFS case, these operational conditions lead to VFA

starvation of PAOs in the anaerobic phase. Consequently, PAOs growth is limited along the aerobic phase, thereby provoking their wash out from the reactor. Higher SRT can support both nitrification and phosphorus removal. However, ammonia is removed via nitrification-denitrification, limiting the nitrogen availability for downstream algal cultivation. Therefore, it can be concluded that the optimal operation ranges between 3.5 and 5 days SRT, where the maximum recovery is reached at SRT 4.5 days. According to Fig.6a the higher Q_P is the higher the phosphorus recovery is. In this scenario, phosphorus recovery is limited by the nitrogen content of the influent, as larger phosphorus recovery would lead to a nitrogen imbalance that could only be corrected by adding an external nitrogen source into the EBP2R effluent. An N-to-P ratio of 17 can be achieved by diverting 28% influent flow as P-stream at SRT=4.5 d. In order to gain some flexibility to better control the system, Q_P is decreased yielding to a higher N-to-P ratio in the effluent. The targeted phosphorus recovery is then 72% of the influent phosphorus. This deviation from the true optimal allows decoupling the control of the phosphorus load from the control of the N-to-P ratio.

For both systems there are up to four candidates to controlled variables (CVs). The phosphorus load and the SRT should be controlled to maximize phosphorus recovery. In addition, the effluent N-to-P ratio should be controlled to meet the nutrient requirements of algal growth. Finally, the dissolved oxygen (DO) should be controlled in the aerobic phase. The available actuators are different for each case. For the SBR, actuators are the effluent pump and the oxygen supply, modelled through the k_La. Additionally, each of the actuators can be active at different times along the cycle, meaning that the same actuator can be used for more than one control purpose, unless the actuator is simultaneously needed by more than one control action. Finally, the N-stream can be split by a valve in two different streams, one feeding the PBR and a second one feeding the CANR system. The CFS has the flow rates of three different streams as manipulated variables, represented by valves in Fig. 7. However, for the CFS the actuators are constantly working. Therefore, each actuator can only be paired with one controlled variable. Since the mixing is not modelled it is not considered as a suitable actuator in this study.

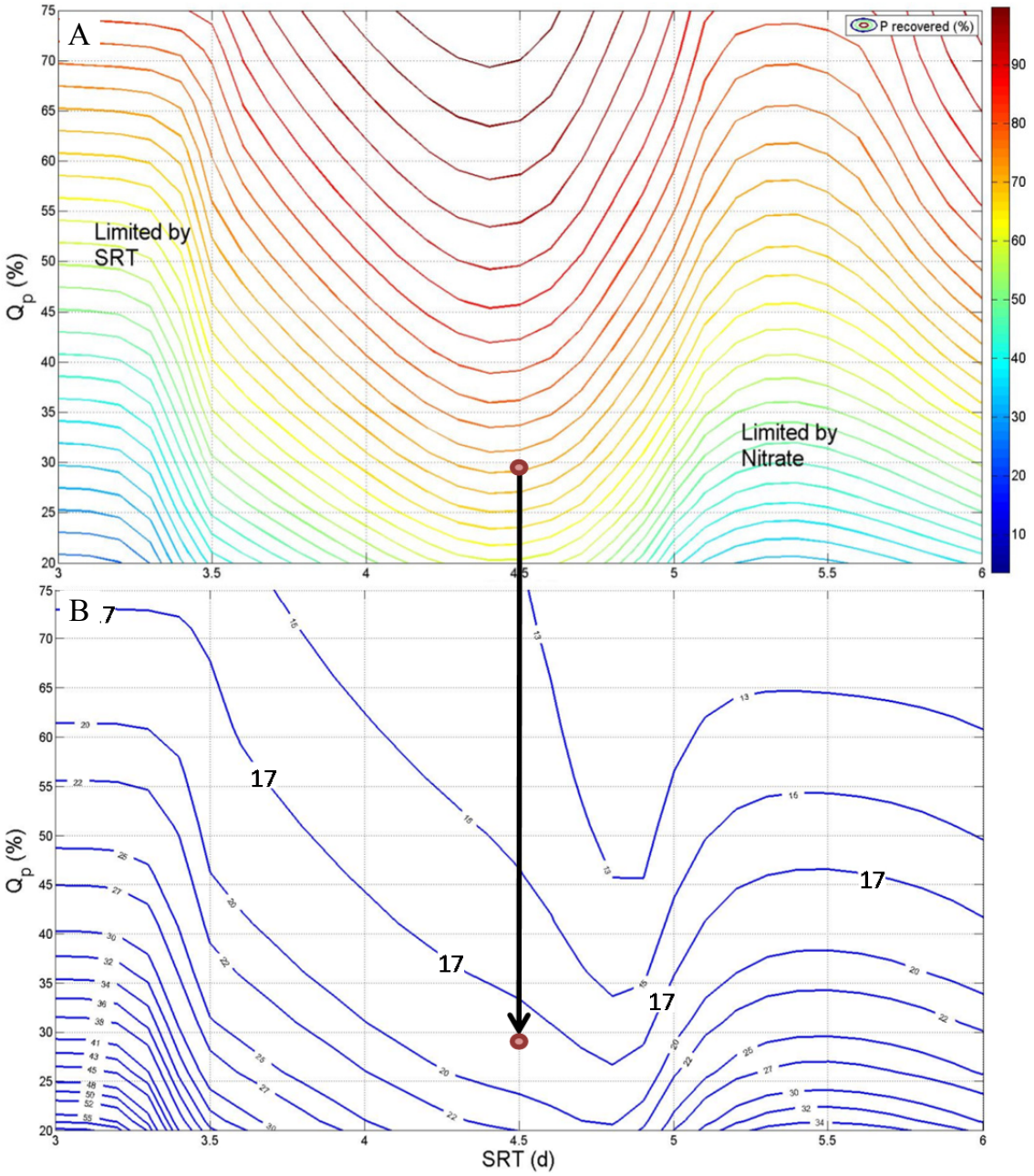

Figure 6: Process performance of the sequencing EBP2R system: a) Percentage of P recovered from the influent as a function of the P-stream flow rate (Q_P) and the SRT; b) N-to-P molar ratio in the EBP2R effluent as a function of the Q_P and the SRT (**paper II**).

For the CFS, Q_P and the wastage flow rate (Q_W) are used to control the phosphorus load conveyed to the PBR and the SRT, respectively. The reminder part of the influent forms the N-stream. This stream is split by a valve, so the valve controls the N-to-P ratio to the PBR by regulating the nitrogen load fed into it. The k_La is used to keep the oxygen level at 1.5 mg/L in the aerobic tank (**paper I**). Fig. 7 shows the resulting control structure.

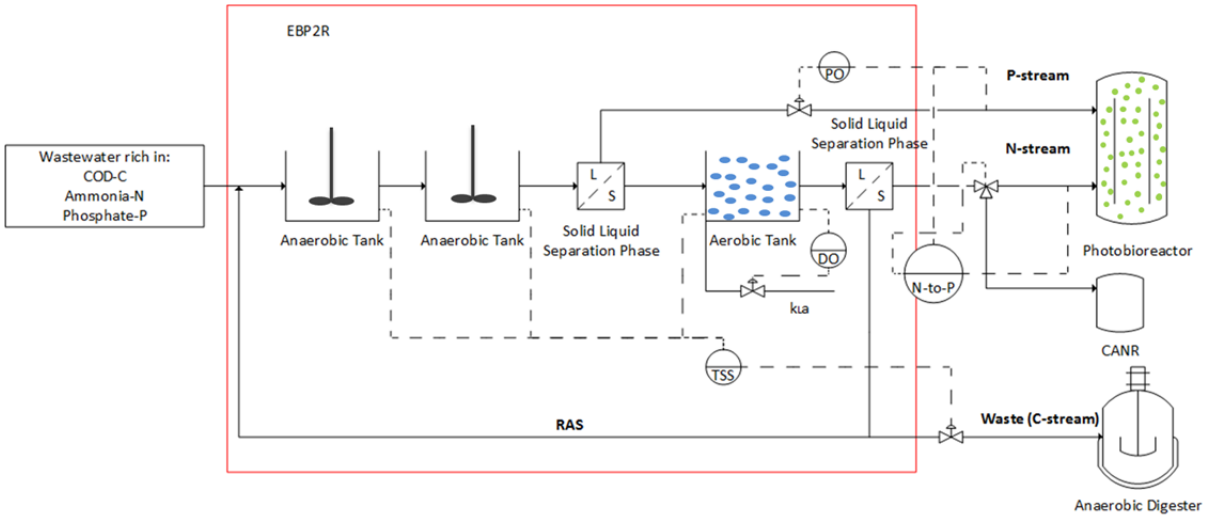

Figure 7: Layout of the control strategy for the continuous flow TRENS. RAS stands for recirculation of activated sludge; CANR stands for completely autotrophic nitrogen removal (**paper II**).

In order to uncouple the control action of phosphorus recovery from that of the N-to-P ratio, the former one should be controlled via Q_P and the second one by the valve splitting the flow between the PBR and the CANR. The SRT is controlled by pumping the needed volume of activated sludge slurry at the end of the second settling phase. The DO level is controlled by manipulating the k_La, as in the CFS configuration. As the oxygen is supplied along the aerobic reaction phase, the control action should take during the batch. The DO is set at 1.5 mg/L (**paper I**). The described control structure is shown in Fig. 8.

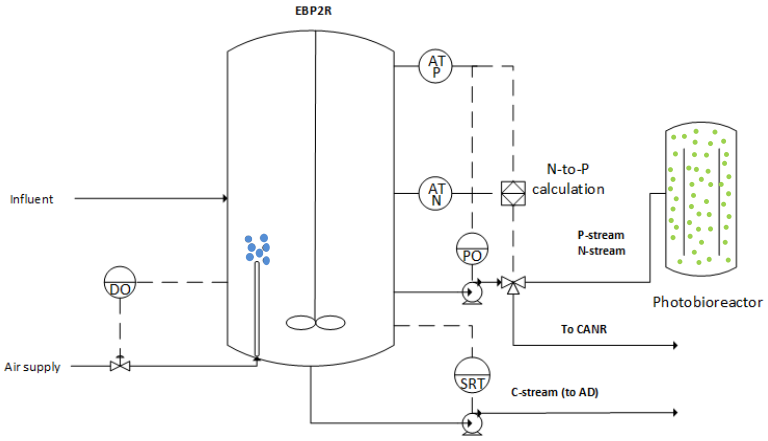

Figure 8. B) Layout of the control strategy for the SBR. CANR stands for autotrophic nitrogen removal and AD stands for anaerobic digestion (**paper II**).

Next task, step 4, is the control tuning, which is made according to the IMC guidelines in both cases (Seborg et al., 2004). The DO controller for the SBR, which is working during the batch, is the only controller that is manually tuned. Since the operation along the batch is dynamic the system cannot be modelled by conventional state-space models used for control design.

Finally, the process control scheme is assessed under dynamic conditions (step 6). Fig. 9a shows that the control system for the CFS is able to smooth the variations on the P-load, only allowing very small deviations from the set point. However, the N-to-P ratio shows some variability (average N-to-P ratio 16.45±2.48, Fig. 9b). When the influent nitrogen into the treatment plant is too low, the control system is not able to keep the set point and the N-to-P ratio falls under the optimal value. Nevertheless, the performance of the EBP2R is comparably better than in the case the system is in open loop.

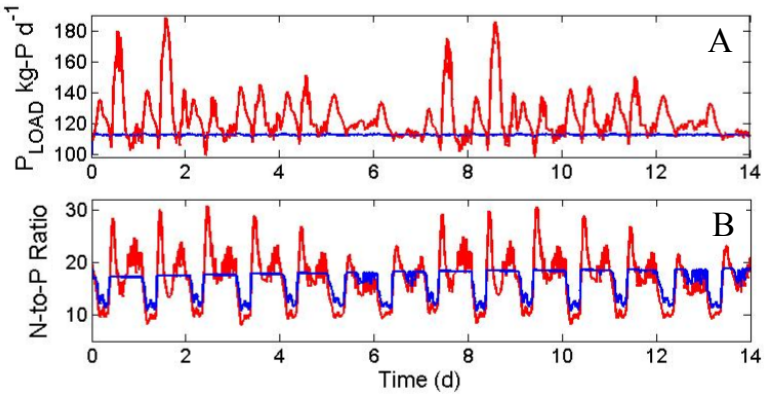

Figure 9. a) P-load fed to the PBR; b) N-to-P ratio fed to the PBR using dynamic input disturbances in the CFS Configuration of the EBP2R. The process response under controlled conditions (blue) and the process response without control (red) are presented (**paper II**).

In the case of the SBR, a retention tank is included in the upstream. The retention tank accumulates the influent, which is continuously received by the WWTP, until the SBR is ready for a new batch. The retention tank is modelled as suggested by Pons et al. (2004). Despite the buffer effect from the upstream retention tank, the dynamic influent disturbance can significantly impact SBR performance. The controller suffers from oscillations, especially due to a peak on phosphorus during the first day of simulation (Fig. 10a). The over-compensation of the controller suggests that it has been tuned too aggressively. However, the system stabilizes along the simulation period, effectively smoothing the phosphorus load. The tuning of the phosphorus load

controller is therefore a critical step on the design of an optimal control structure for the SBR configuration. With regard to the N-to-P ratio (Fig. 10b), the system effectively keeps the effluent quality (average N-to-P ratio is 16.9±0.07).

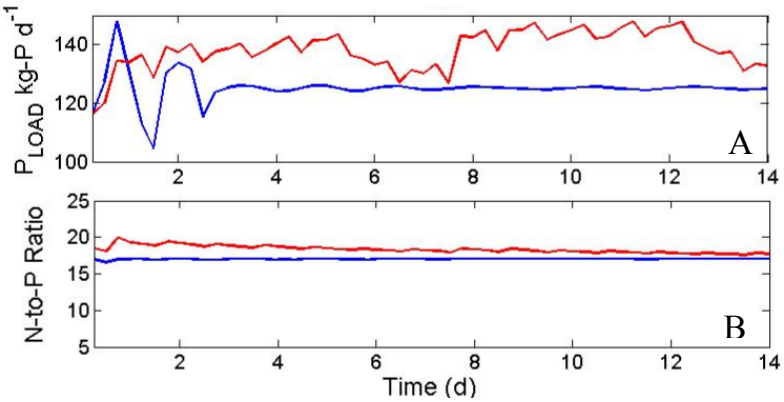

Figure 10. a) P-load fed to the PBR; b) N-to-P ratio fed to the PBR using dynamic input disturbances in the SBR Configuration of the EBP2R. The process response under controlled conditions (blue) and the process response without control (red) are presented (**paper II**).

2.4 Laboratory scale demonstration of short SRT EBPR

After demonstrating the process feasibility through the model based studies (i.e. **papers I** and **II**) the operational conditions were tested in the lab. This step is important, as there are examples in literature showing that model-based optimization may induce changes on the reactor microbial communities that the model cannot capture, leading to falsification issues (Sin et al., 2006). Both, the SBR and the CFS were implemented in laboratory-scale and operated as a low SRT EBPR system (i.e. as an EBP2R with Q_P=0; **paper III**).

2.4.1 Sequencing batch reactor

An 8 L SBR reactor (Fig. 11) was inoculated with activated sludge from Lynetten WWTP, which works as a conventional EBPR system. The reactor was operated for 190 days at three different SRT, treating wastewater from Lundtofte WWTP. The system was operated for 50 days at SRT 8 days, supporting nitrification-denitrification and biological phosphorus removal. At day 51 the SRT was shifted to 4 days and nitrifying bacteria were effectively washed out from the system, indicated by the negligible effluent nitrate con-

centration (Fig. 12A). Surprisingly, the SBR suffered extreme filamentous bulking. The sludge volume index (SVI) increased up to 1100 ml/g. Along with the deterioration of the sludge settling properties, the phosphorus removal considerable decreased. Analysis through quantitative fluorescence in situ (qFISH) analysis revealed a decrease in *Accumulibacter* population (Fig. 11).

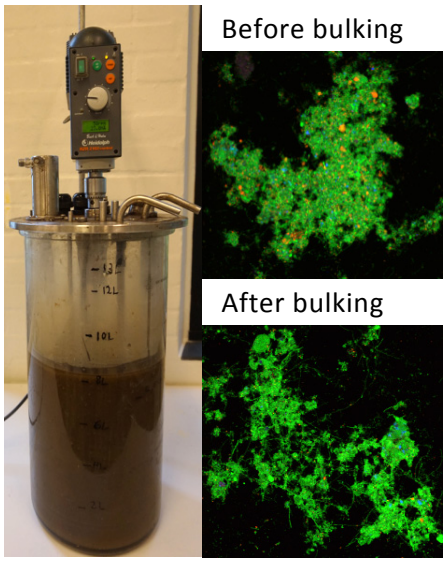

Figure 11. Lab scale EBPR reactor operated as an SBR (left side). FISH pictures targeting PAOs: red probe targets *Accumulibacter* bacteria; blue probe targets *Tetrasphaera* (**paper III**).

Yamamoto-Ikemoto et al. (1991) found similar bulking events in EBPR systems provoked by *Thiothrix*. *Thiothrix* proliferated in those reactors thanks to the synergic interactions with SRBs. In their study, SBRs were reducing sulphate to reduced sulphur compounds, such as sulphide, that serve as substrate for *Thiothrix*. Sulphate reduction along the anaerobic phase was then monitored in the laboratory scale SBR, revealing that up to 25 mg-SO4/L were reduced. In order to phase SRBs out the anaerobic SRT was decreased from 1.2 days down to 0.68 days. Consequently, the sulphate reduction stopped and the SVI decrease to ~200ml/g. Phosphorus removal was restored to previous levels, ranging from 85-99%. However, AOB actively oxidized ammonia to nitrite between days 132 and 156. Therefore, in an attempt to wash the AOB out of the SBR, the system SRT was shifted from 3.5 d to 3 d. Complementary, the oxygen level was maintained between 2 and 3 mg/L, which was found enough to support phosphorus removal by PAOs, but not ammonia oxidation by AOB (in agreement with **paper I**). This strategy effectively

suppressed AOB activity and ammonium became the main nitrogen form in the effluent. The short SRT EBPR was kept stable for 35 more days. SVI was relatively stable between 200 and 300 mg/L, suggesting that moderated bulking conditions could not be avoided in the short SRT EBPR. The 25% of the ammonia was removed via microbial assimilation and phosphate was effectively removed leading to phosphate concentrations below 1 mg-P/L in the effluent.

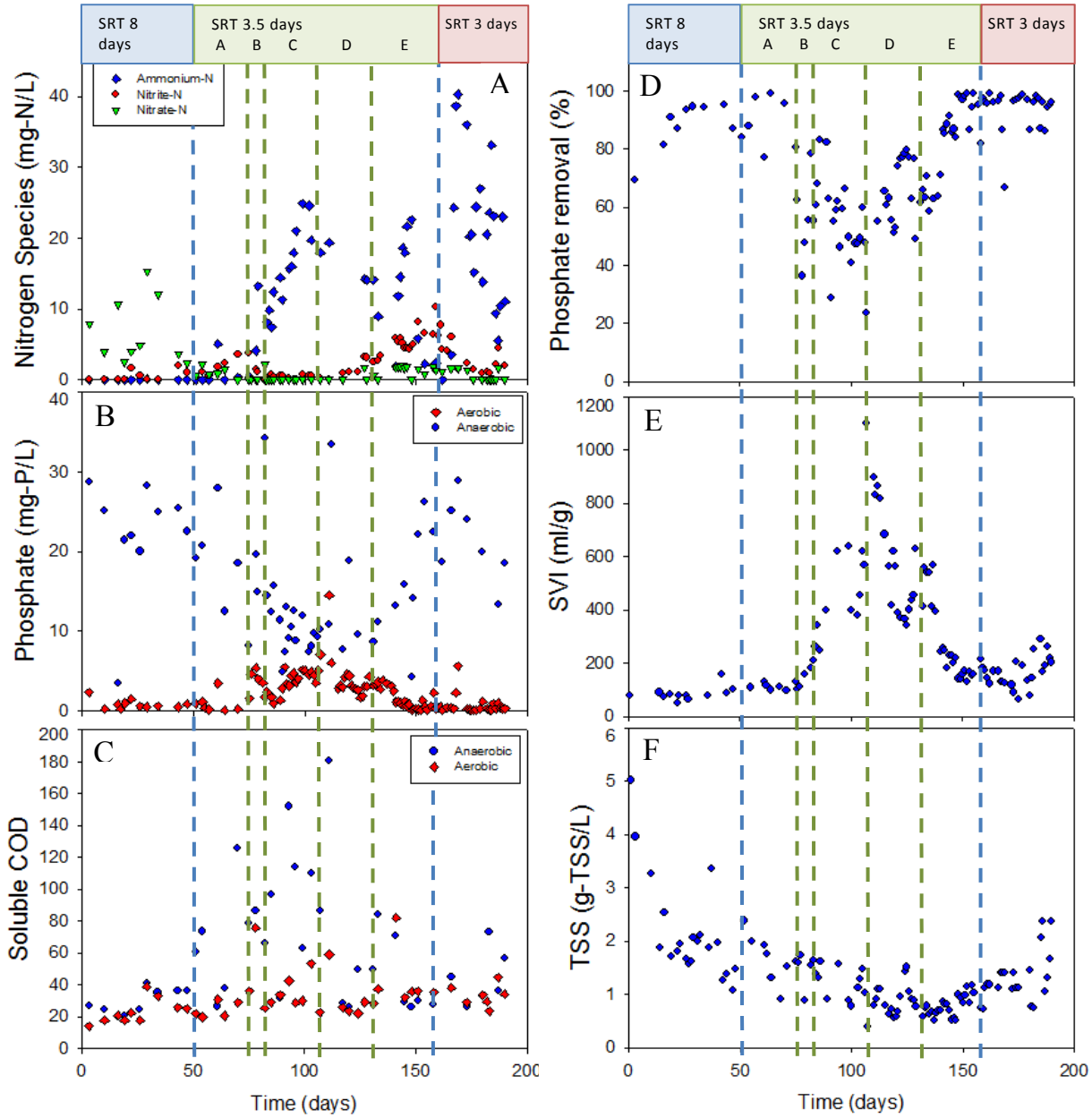

Figure 12. SBR reactor performance through 190 days. a) Ammonia, nitrite and nitrate at the end of the aerobic phase; b) phosphate at the end of the aerobic and anaerobic phases; c) soluble COD at the end of the anaerobic and aerobic phases; d) phosphate removal; e) sludge volumetric index; f) total suspended solids. Phase A: from day 50 to day 78; phase

B: from day 78 to 83; phase C: from day 83 to 109; phase D: from day 109 to day 132; phase E: from day 132 to day 156 (**paper III**).

After SRT was shifted to 3.5 days, microbial community analysis via 16S rRNA amplicon sequenced revealed that *Thiothrix* species significantly increased their abundance in the reactor (Fig. 13). *Thiothrix* reached highest relative abundance (around 90%) when 25 mg/L of sulphate were reduced during the anaerobic phase. These two evidences suggest that SRBs were actively reducing sulphate, although we could not find them through our molecular analysis (data not shown). As previously introduced, two different types of SRBs may grow in the reactor (Hao et al., 2014). Incomplete oxidizing SRBs, which oxidize the organic substrates, such as propionate, to acetate, which is then available for other bacteria, like PAOs. This type of SRBs was found to enhance phosphorus release by providing acetate to PAOs (Yamamoto-Ikemoto et al., 1998). Complete oxidizing SRBs, which can completely oxidize organic substrates, such as propionate, to carbon dioxide. This SRB type may compete with PAOs for propionate, thereby upsetting the EBPR performance. Furthermore, the production of sulphide during the anaerobic phase may have inhibited relevant processes for phosphorus removal, like phosphorus release by PAOs or fermentation of complex substrates (McCartney and Oleskiewicz, 1991; Saa et al., 2013).

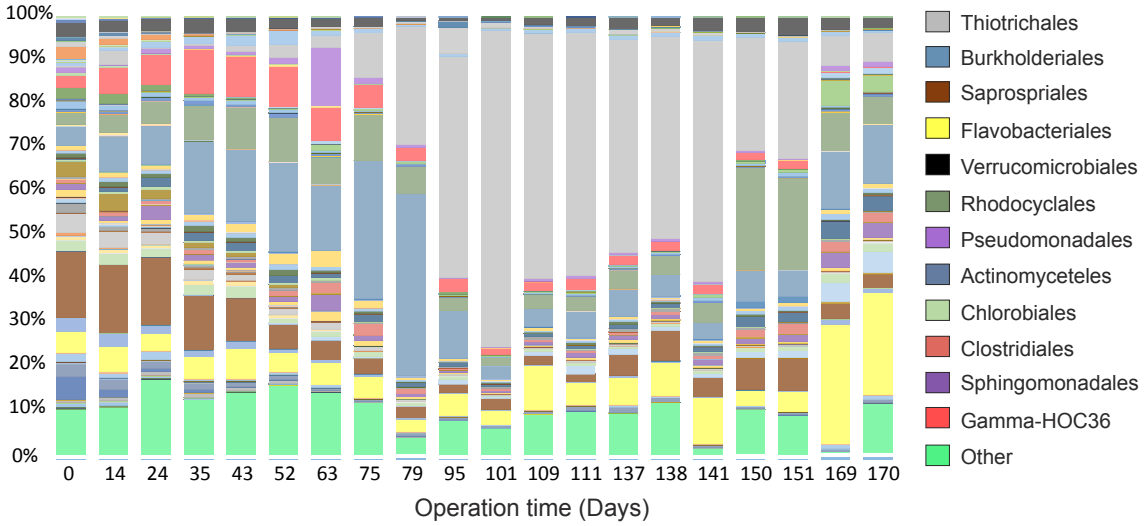

Figure 13: Order-level taxonomic classification of 16S rRNA amplicons at selected time points of the reactor operation. Taxa abundance is expressed in percentage (**paper III**).

2.4.2 Continuous flow system

The EBP2R was built as a sequence of glass-made continuous stirred tank reactors (Fig. 14). During the operation time, the system was run as an EBPR system (i.e. P-stream diversion was 0).

The system was run for 50 days at SRT 8 days. SVI, sulphate reduction and P-removal data are shown Fig. 15. The general trend was that phosphorus removal was effective and sludge was settling well during the first 30 days. Both SVI and phosphorus removal were unstable in the last 23 days. The system performance seemed to peak around day 20, and slowly degenerated until day 30, from where the performance became more unstable. The sulphate concentration was measured in the last half of the experiment (Fig. 15a). Sulphate reduction was occurring in the anaerobic reactor. On day 29 and day 39 the volume in the anaerobic reactors were decreased 2.2 and 1.9 litres, respectively, in an attempt to wash out SRBs, as they can only grow under anaerobic conditions. Nevertheless, Fig. 15A shows that this mitigation strategy was not very effective as sulphate was still partially reduced. Settleability, however, had stabilized from day 49, suggesting that the reminder sulphur reduced compounds were not enough to support *Thiothrix* growth. Phosphorus removal could not be restored and the system was stopped.

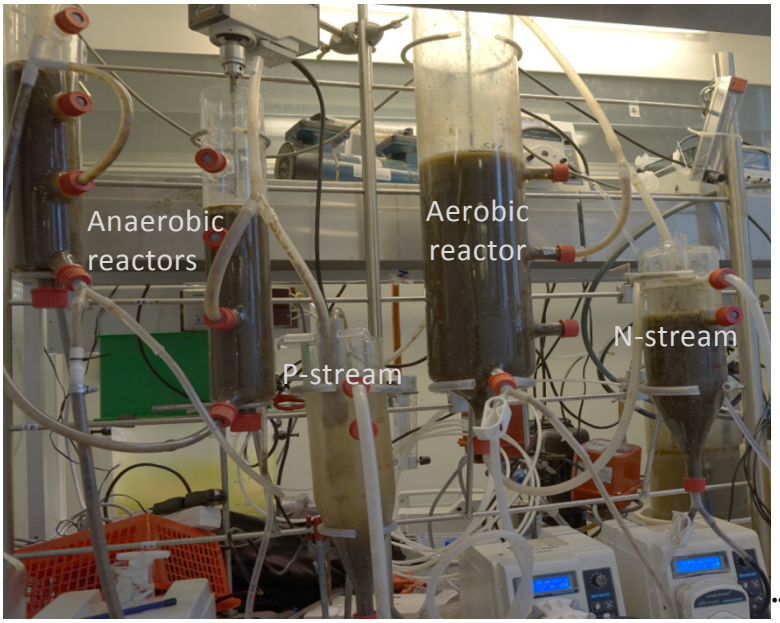

Figure14. Continuous flow system layout.

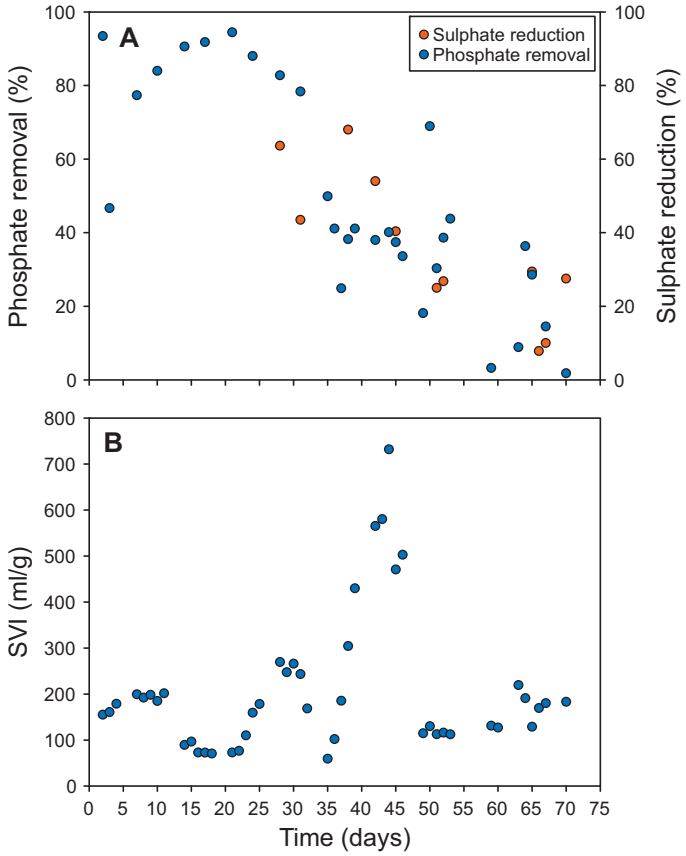

Figure 15. Continuous flow system performance: A) phosphate removal and sulphate removal; B) sludge volume index.

Thiothrix were targeted with qFISH analysis and found in abundances up to 60% of total microbial population (Fig.16). *M. parvicella* was also found in relatively high abundances (up to 12% of total bacteria).

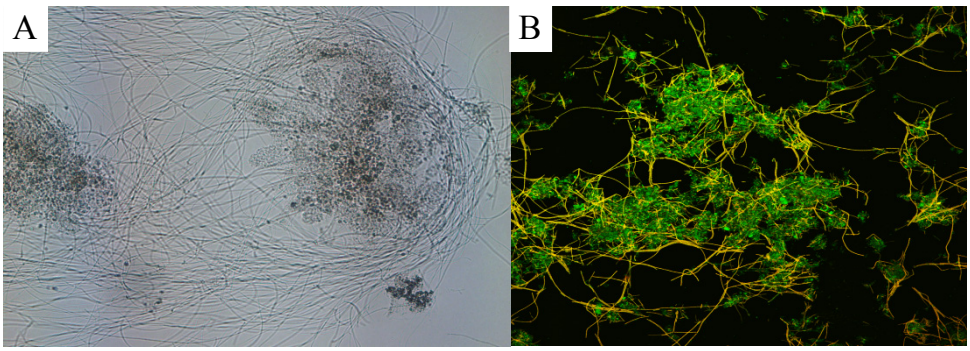

Figure 16: Filamentous bacteria grown in the continuous flow systems. A) Bright field microscopy image; B) FISH pictures targeting *Thiothrix*.

Results obtained in this study suggest that the bacterial populations found to drive the functionality of the short SRT EBPR should – in addition to the ecological model proposed by Nielsen et al. (2010) – account for the SRBs as potential inhibitors of the biological phosphorus removal. The competition, synergy and inhibition pathways are summarized in Fig. 17.

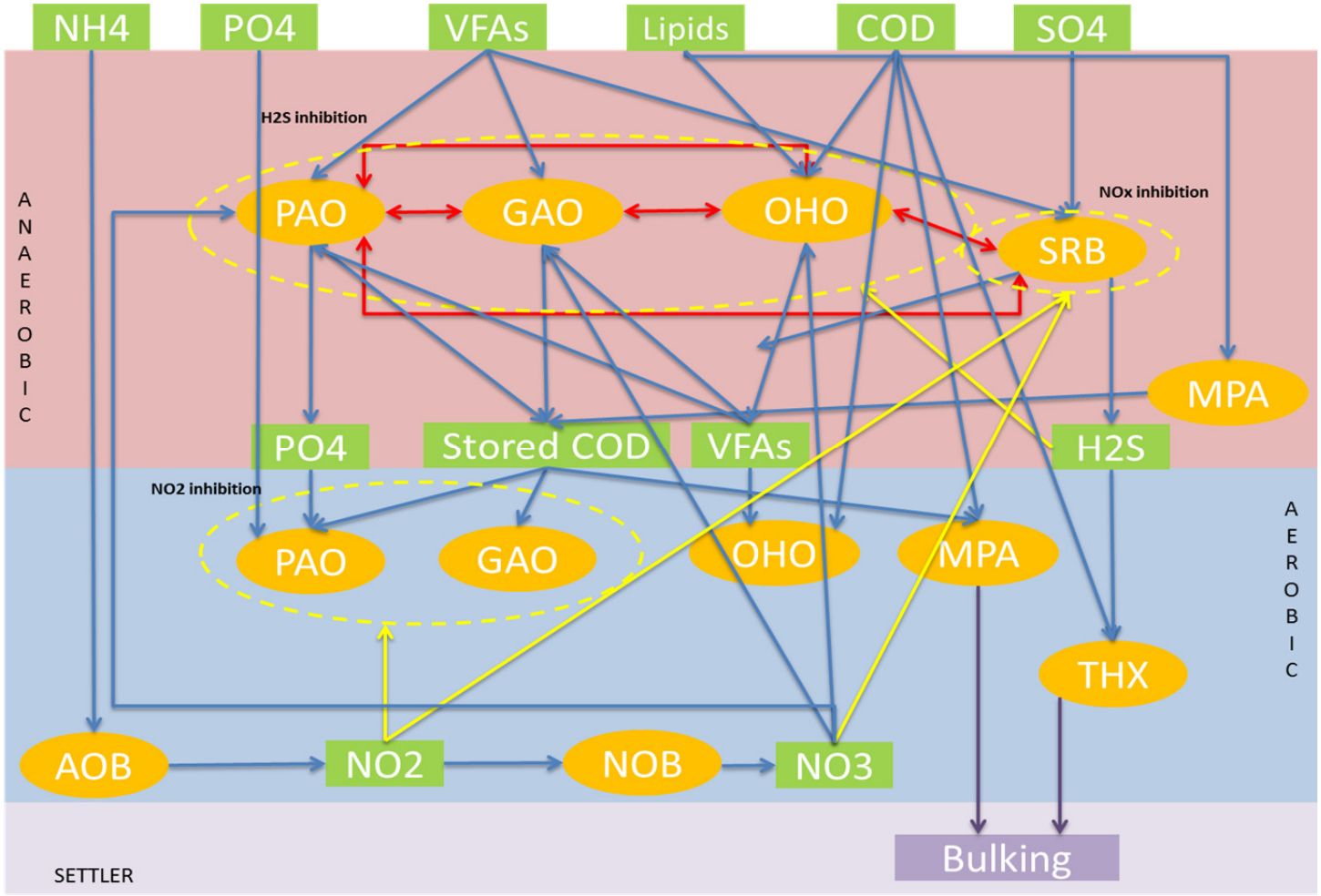

Figure 17: Proposed ecological model for short SRT EBPR systems. Blue: substrates flows; Red: competitions for VFAs; Yellow: inhibition of bacterial activity; Purple: filamentous bulking.

3 Green microalgae for resource recovery

Algae are phototrophic eukaryotes that exist as both microorganisms and macrooganisms. Algae can grow both photoautotrophically or heterotrophically, depending on the environmental conditions. Out of microalgae, chlorphytes is one of the largest phyla, which is characterized by a green colour due to the chlorophyll content in their chloroplasts (reason they are also referred to as green microalgae). Green microalgae are abundant in a wide range of environments, from fresh water or marine environments to moist soil, snow or inside rock pores (Andersen, 2005; Madigan et al., 2009). Green microalgae can also grow in wastewater taking up and storing inorganic nutrients, thereby facilitating nutrient recovery. In the work reported in this thesis *Chlorella sp.* and *Scenedesmus sp.* have been chosen as model algae.

3.1 Cultivation conditions

Algal growth is influenced by many factors. Light supply is one of the most studied factors, as the type and intensity of the light source will affect the algal growth and biomass composition. Light serves as energy source in the photosynthesis, reaction whereby algae assimilates inorganic carbon and water yielding to biomass and oxygen. The photosynthesis is split in two reactions, both taking place in the chloroplast (Fig.18) : i) the light reactions, whereby the algae uses light to produce energy and water (Eq.5); and ii) the dark phase, also known as the Calvin cycle, whereby the algae uses the produced energy to fixate carbon dioxide as phosphoglycerate (Eq.6). In the light reaction two electrons are donated from water, resulting in the production of oxygen. The electrons are transferred through a chain of electron carriers, referred to as "Z-scheme", all of them located in the thylakoid membrane (Richmond, 2004). The light reaction provides the biochemical reductant $NADPH_2$ and chemical energy in form of ATP. Both the $NADPH_2$ and ATP are used for inorganic carbon assimilation, a process that is carried out in the stroma, which is then used for cellular growth (Wilhelm & Jakob, 2011). Phosphoglycerate, product of the Calvin cycle, is an intermediate of the glycolysis, from where any other biomolecule needed by the algae can be built.

$$2\,NADP + 3\,H_2O + 2\,ADP + 2P_i \xrightarrow{hv+Chla} 2\,NADPH_2 + 3\,ATP + O_2 \quad (5)$$

$$CO_2 + 4\,H^+ + 4\,e^- \xrightarrow{2\,NADPH_2,3ATP} (CH_2O) + H_2 \quad (6)$$

Fig. 18 is an illustration of the algal metabolism where the links between photosynthesis and the other metabolic pathways are shown.

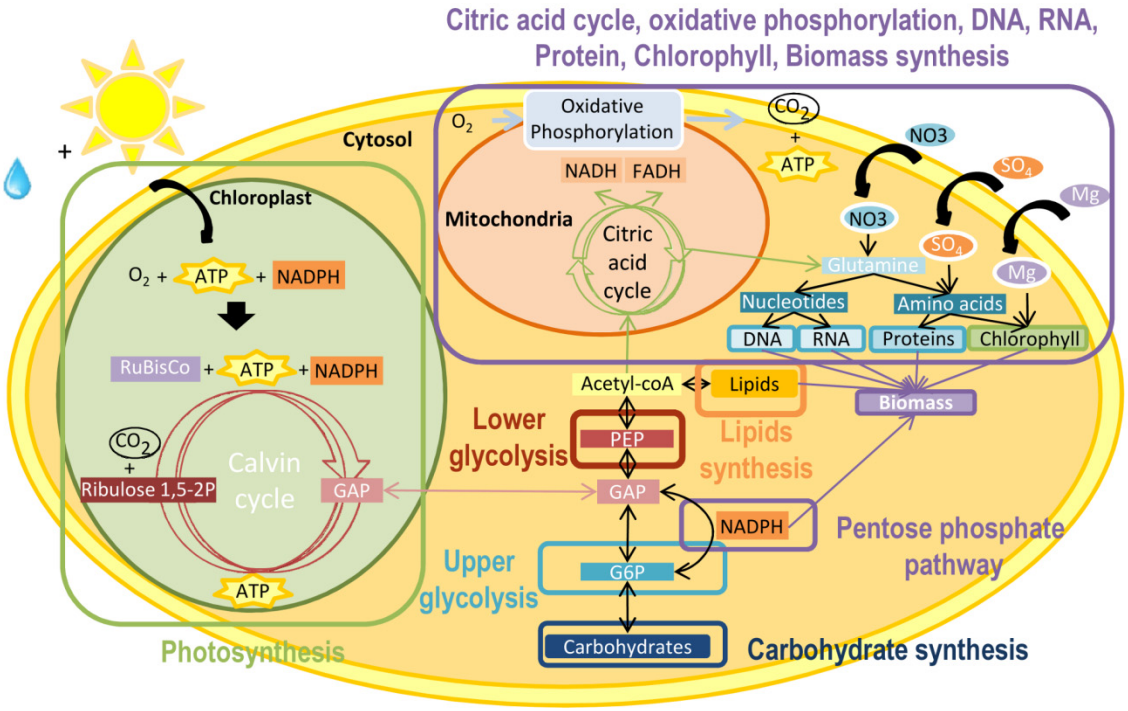

Figure 18. Simplified central carbon metabolic network of unicellular photoautotrophic algae. Taken from Baroukh et al. (2014).

The impact of light on algal cultivation depends on the algae specie. Nevertheless, there are some general aspects common to any algae. As example, algal growth rate tends to increase with light intensity until a maximum is reached, whereby the algae cannot utilize more photons for photosynthesis. The extra energy received from light is dissipated as heat. Beyond the optimal light intensity algal growth rates may decrease due to light inhibition. Furthermore, light is heterogeneously distributed along the PBR. When light travels along the PBR, it can be absorbed by pigments, mainly chlorophyll, or reflected on the cell and reactors wall resulting in light scattering (Wágner et al., 2014). Light intensities may also affect the algae composition. As an example, at high light intensities algae tend to produce more pigments for light protection (Seyfabdi et al., 2011). Other example is the effect on lipid composition. Van Wagenen et al. (2012) found that low light intensities increase the relative abundance of unsaturated fatty acids. Taken together, the selection of light source, either natural or artificial, and light intensity is not a triv-

ial choice and must be considered to properly optimize the PBR performance (Grognard et al., 2014). Furthermore, the PBR design should consider the light attenuation across the reactor due to self-shading and minimize it in order to maximize algal productivity.

Algae can grow on the different species from the carbonate system, namely, carbon dioxide, bicarbonate and carbonate. Algae have higher affinity for carbon dioxide than for bicarbonate (Decostere et al., 2013), while they show the lowest affinity for carbonate (Yeh et al., 2010). The consumption of carbon dioxide leads to a pH increase. However, the supply of carbon dioxide through bubbling usually neutralizes the uptake effect on pH. If carbon dioxide is not supplied and algal growth only relies on the assimilation of bicarbonate, pH may considerably increase as consequence of the proton consumption.

If the culture is in complete darkness, algae can also grow heterotrophically on different organic carbon sources, such as glucose or acetate (Pérez-García et al., 2011). As algae cannot grow on anaerobic conditions, regardless the carbon source, oxygen has to be supplied. In the presence of both light and organic carbon sources some algae can grow mixotrophically, thereby using both carbon dioxide and organic carbon as substrate (Van Wagenen et al., 2015). Organic carbon molecules undergo metabolic routes common to other microorganisms, such glycolysis or glyoxylate cycle, thereby producing energy and biomass (Pérez-García et al., 2011).

Algae are able to use different nitrogen sources for growth, being the most commonly reported ammonia, nitrate and urea (CH_4N_2O). Ammonia can be assimilated and directly converted to biomolecules, making it the preferable nitrogen source for most of the green microalgae (Cai et al., 2013). Nitrate and nitrite are reduced by nitrate and nitrite reductase enzymes, producing ammonia and using pyridine nucleotide as an electron donor. Nitrate uptake is a continuous process, while nitrite reduction to ammonia only takes place under light exposure. Therefore, under light limitation nitrite may accumulate in the cytoplasm, which is toxic for algae, and may be excreted back to the media (Malerba et al., 2012). If nitrate level is too high (e.g. up to 170 mg-N/L for *Chlorella sp.*) nitrite can accumulate even in non-light limited cultures, thereby inhibiting algal growth (Jeanfils et al., 1993). Finally, the nitrogen source taken up by the algae will also affect the pH. If ammonia is being taken up the pH will decrease due to the release of protons, while if nitrate is taken up pH will rise (see e.g. Podevin et al., 2015).

Microalgae consume phosphorus to build different cellular structures and biomolecules. Under certain conditions, algae can uptake more phosphorus than anabolically required. On the one hand, algae can increase their phosphorus uptake rate if they are cultivated in a phosphorus rich medium after phosphorus starvation (Brown and Shilton, 2014). On the other hand, algae can store phosphorus within the biomass as polyphosphate, defined as a luxury phosphorus uptake (Powell et al., 2008). Polyphosphate can be present as acid-soluble polyphosphate, which is easily available for metabolism, and acid-insoluble polyphosphate, which is a solid fraction that the algae consume once phosphorus becomes limiting in the medium (Powell et al., 2009). While this process is not common in natural environments, such as lakes or sea, it can take place in wastewater, where phosphorus concentrations are rather high.

The optimal N-to-P ratio is the ratio at which the nutrient limitation shifts from one nutrient to the other. According to Rhee and Gotham (1980), the optimal N-to-P ratio for each alga can be estimated as the relation between the minimal nitrogen and phosphorus storage at growth rate zero. The scientific community has taken as reference the Redfield ratio, reported as the average optimal nutrient by the oceanic phytoplankton, which value is 16 (Redfield, 1958). However, depending on the algal species the N-to-P ratio ranges from 8 (Christenson and Sims, 2011) up to 100 (Geider and La Roche, 2002). Furthermore, the optimal N-to-P ratio may vary depending on a number of factors, such as light, temperature, salinity and nutrient content (Rhee and Gotham, 1980; Beuckles et al., 2015). To keep an optimal nutrient balance is of vital importance when the intended use of green microalgae is wastewater treatment. In case there is a nutrient imbalance, algae will completely remove the limiting nutrient while the nutrient supplied in excess will not be completely taken up (see e.g. Tuanet et al. 2014). Additionally, the N-to-P ratio drives the competition between different algae species in mixed cultures and helps to keep stable pure cultures (Richmond, 2004).

Apart from macronutrients, like nitrogen and phosphorus, microalgal growth depends on a wide number of micronutrients, mainly minerals and metals, as potassium, magnesium, iron or copper. While these micronutrients are essential for microalgal growth they may become toxic at high concentrations (Cai et al., 2013).

pH may impact the algal growth in different ways. On the one hand, pH influences the distribution of inorganic nutrients and their solubility in the me-

dium, thereby affecting the substrate availability for algal growth (Cai et al., 2013). On the other hand, pH may directly inhibit algal growth and can even be lethal depending on pH levels reached (Berge et al., 2012). Algal growth can also affect pH depending on the substrate used for growth, as detailed in the previous paragraphs. Therefore, pH control is crucial to keep stable the PBR (Olivieri et al., 2014). If the temperature of the culture increases, faster growth rates may be achieved. However, as side effect, the optimal pH range for algal growth may be reduced (Mayo 1997). Therefore, pH control should also consider the culture temperature.

3.2 Modelling green microalgal growth on used water

3.2.1 State of the art

The models range in complexity, from considering the influence of a single variable on growth, e.g. light intensity (Grima et al., 1994; Huesemann et al., 2013), to incorporating the combined influence of several variables, such as light, nutrient availability, temperature or pH (Ambrose, 2006; Wolf et al., 2007; Quinn et al., 2011; Broekhuizen et al., 2012; Guest et al., 2013; Decostere et al., 2013; Adesanya et al., 2014; Coppens et al., 2014; Fachet et al., 2014). Despite the complexity of these last models is comparably high, they lack important aspects related to microalgal applications. As an example, the biofilm PHOBIA model (Wolf et al., 2007) includes the growth of heterotrophs, nitrifiers and microalgae on inorganic carbon, light and nitrogen, but disregards algal growth dependency on phosphate, making it inefficient for applications in used water treatment. The model by Broekhuizen et al. (2012), accounts for the effects of pH, inorganic carbon, oxygen, nitrogen, phosphate and light on microalgal growth. However, growth and nutrient uptake are considered directly coupled, thereby neglecting the nutrient storage and growth on the stored nutrients. Droop (1973) proposed a model that describes uptake and storage and growth on stored nutrients as two different processes related by the internal nutrient quota. Consequently, the model can describe growth in the absence of external nitrogen or phosphorus. There are several models with multiple substrate limitations, following Droop's approach (Ambrose, 2006; Bernard, 2011; Quinn et al., 2011; Guest et al., 2013; Fachet et al., 2014).

Although growth of algae on different organic substrates is well documented (Perez-Garcia et al., 2011), none of the above mentioned models considers the mixotrophic or heterotrophic growth. Moya et al. (1997), proposed a simple model for microalgal growth as a function of light (autotrophic growth) and acetate (heterotrophic growth) – the latter expressed with the Haldane kinetics. While this approach is useful to predict heterotrophic algal growth in nutrient excess conditions, the effects of both nitrogen and phosphorus, amongst others, are missing.

In literature, there exist three systematic approaches to model the effect of light on algal growth (Béchet et al., 2013): i) type I: models accounting for an average light intensity and its impact on the algal growth; ii) type II: models accounting for the light gradient in the PBR and the effect on the photosynthetic rate; and iii) type III: models that consider the photosynthetic rate of an individual algal cell as a function of the light history. The effect of light on algal growth can be modelled taking into account photo-inhibition using the Steele, Peeters and Eilers or Haldane kinetics (Bouterfas et al., 2002; Ambrose, 2006), or omitting the inhibition by light using the Monod, Platt and Jassby, Poisson single-hit models or Smith equations (Bouterfas et al., 2002; Ambrose, 2006; Skjelbred et al., 2012).

3.2.2 Green microalgal growth model in the activated sludge framework: the ASM-A

Despite the wide literature on algal modeling, none of the proposed models fully address the applicability of algae in used water treatment. While for bacterial based systems there are already consensus models, such as the ASM model family (Henze et al., 2000), for algal based systems there is still a need to unify modeling approaches. Such a consensus model to predict green microalgal activity has the potential to be a versatile tool for process design, control and optimization. Furthermore, such a novel model should be consistent in terms of units, nomenclature and state variables with the already existing ASM models. Otherwise, the interfacing between algal models and activated models may become a tedious task, hindering the implementation of PBR models as part of the plantwide models already existing (e.g. Benchmark Simulation Model 2, Nopens et al., 2010). **Paper IV** aims to identify and evaluate a process model complying with the aforementioned requirements. The model is developed as an extension of the ASM-2d (Henze et al., 1999), which considers all the bacteria and compounds involved in long SRT EBPR systems. However, **paper IV** focuses only on the algal processes.

Uptake and storage of nitrogen (R1 and R2): ASM-A considers the microalgal uptake and storage of both ammonia (R1) and nitrate (R2) nitrogen (Table 1). The uptake and storage of nitrogen is dependent on the availability of external nitrogen (S_{NH4} or S_{NO}), as well as the internal cell quota of nitrogen ($X_{Alg,N}$), defined as total cell internal storage of nitrogen. Nitrogen uptake rate decreases as the stored nitrogen approaches the maximum internal cell quota, $X_{Alg,Nmax}$, in the biomass (X_{Alg}). As explained before, ammonia is preferred over nitrate for most algal species (Cai et al., 2013). Hence an inhibition term for nitrate uptake is included when ammonia is available (R2, Table 1).

Uptake and storage of phosphorus (R3): The uptake and storage of phosphorus (R3, Table 1) is dependent on the availability of external soluble orthophosphate in wastewater (S_{PO4}), and on the internal cell quota of phosphorus ($X_{Alg,PP}$), defined as total cell internal storage of phosphorus. Phosphorus uptake rate decreases as the stored phosphorus approaches the maximum internal cell quota, $X_{Alg,PPmax}$.

Photoautotrophic growth (R4): Nutrient limitations are described as suggested by Droop (1973). Therefore, growth is considered to be dependent on the stored nitrogen and phosphorus rather than on the bulk liquid concentrations. The specific growth rate decreases as the internal cell quota approaches the minimum subsistence cell quota ($X_{Alg,Nmin}$ or $X_{Alg,PPmin}$). The consumption of inorganic carbon (S_{Alk}) is modelled using Monod kinetics. Light limitation is determined by the photo-synthetically available irradiance passing through the PBR. It is assumed that the microalgae are exposed to a constant average light intensity (type I light model, Béchet et al., 2013), denoted as I_{Av}. Light dependence is modelled using the Steele equation.

Heterotrophic algal growth (R5): In accordance with the ASM-2d, acetate is used as the organic carbon substrate (S_A). The Monod kinetics was fitted to model the heterotrophic growth as a function of the substrate concentration. Oxygen serves as a terminal electron acceptor for heterotrophic growth (S_{O2}), and its effect follows the Monod kinetics. Inhibition of the heterotrophic growth by light intensity is modelled using the competitive inhibition term. Growth dependence on nutrients is modelled as for the phototrophic growth.

Algal decay (R6): The algal decay process includes the internal resources used for maintenance, biomass loss during dark respiration and death and lysis that will reduce the amount of active biomass in the culture. In addition, the term includes reduction in biomass due to predators grazing on the algal biomass. The decay process is modelled following the dead-regeneration principle, which states that a fraction of the products from decay become available for microbial growth (van Loosdrecht and Henze 1999).

Table 1: The Gujer matrix of ASM-A model including the state variables (**paper IV**):

Component	NH_4	NO_3	Internal quota N	PO_4	Internal quota P	Inorg. carbon	Acetate	O_2	Algal Biomass	Inert Particulates	Slowly biodegradable Particulate	Rate
Symbol Unit	S_{NH4} gN/m^3	S_{NO} gN/m^3	$X_{Alg,N}$ gN/m^3	S_{PO4} gP/m^3	$X_{Alg,PP}$ gP/m^3	S_{Alk} $g\ C/m^3$	S_A $gCOD/m^3$	S_{O2} $gCOD/m^3$	X_{Alg} $gCOD/m^3$	X_I $gCOD/m^3$	X_S $gCOD/m^3$	
Process	**Stoichiometric Matrix**											
Uptake and storage of nitrogen from NH4	**-1**		**1**									**R1**
Uptake and storage of nitrogen from NO3		**-1**	**1**									**R2**
Uptake and Storage of PO4				**-1**	**1**							**R3**
Autotrophic growth			**$-iN_{Xalg}$**		**$-iP_{Xalg}$**	**$-1/Y_{Xalg,SAlk}$**		**$2.67/Y_{Xalg,SAlk}$**	**1**			**R4**
Heterotrophic growth			**$-iN_{Xalg}$**		**$-iP_{Xalg}$**	**$0.4/Y_{Ac}$**	**$-1/Y_{Ac}$**	**$1-1/Y_{Ac}$**	**1**			**R5**
Decay	**$iN_{Xalg} - fX_I.iN_{XalgI} - (1-fX_I).iN_{XalgS}$**			**$iP_{Xalg} - fX_I.iP_{XalgI} - (1-fX_I).iP_{XalgS}$**				**$-(1-fX_I)$**	**-1**	**fX_I**	**$1-fX_I$**	**R6**

Process rates	
R1 [g N m^{-3} d^{-1}]	$k_{NH4} \cdot \frac{S_{NH4}}{S_{NH4} + K_{NH4,Alg}} \cdot \frac{X_{Alg,Nmax} \cdot X_{Alg} - X_{Alg,N}}{X_{Alg,Nmax} \cdot X_{Alg}} \cdot X_{Alg}$
R2 [g N m^{-3} d^{-1}]	$k_{NO} \cdot \frac{S_{NO}}{S_{NO} + K_{NO,Alg}} \cdot \frac{K_{NH4,Alg}}{K_{NH4,Alg} + S_{NH4}} \cdot \frac{X_{Alg,Nmax} \cdot X_{Alg} - X_{Alg,N}}{X_{Alg,Nmax} \cdot X_{Alg}} \cdot X_{Alg}$
R3 [g P m^{-3} d^{-1}]	$k_{PO4} \cdot \frac{S_{PO4}}{S_{PO4} + K_{PO4,Alg}} \cdot \frac{X_{Alg,PPmax} \cdot X_{Alg} - X_{Alg,PP}}{X_{Alg,PPmax} \cdot X_{Alg}} \cdot X_{Alg}$
R4 [g COD m^{-3} d^{-1}]	$\mu_{A,max} \cdot (1 - \frac{X_{Alg,Nmin} X_{Alg}}{X_{Alg,N}}) \cdot (1 - \frac{X_{Alg,PPmin} X_{Alg}}{X_{Alg,PP}}) \cdot \frac{S_{Alk}}{S_{Alk} + K_{Alk}} \cdot \frac{I_{Av}}{I_S} \cdot e^{1 - \frac{I_{Av}}{I_S}} \cdot X_{Alg}$
R5 [g COD m^{-3} d^{-1}]	$\mu_{H,max} \cdot (1 - \frac{X_{Alg,Nmin} X_{Alg}}{X_{Alg,N}}) \cdot (1 - \frac{X_{Alg,PPmin} X_{Alg}}{X_{Alg,PP}}) \cdot \frac{S_A}{S_A + K_A} \cdot \frac{S_{O2}}{S_{O2} + K_{O2}} \cdot \frac{K_I}{K_I + I_{Av}} \cdot X_{Alg}$
R6 [g COD m^{-3} d^{-1}]	$b_{Xalg} \cdot X_{Alg}$

3.2.3 ASM-A model calibration

The model has been calibrated for a green microalgae consortium isolated from a natural pond exposed to wastewater near Barcelona, Spain, in February 2012 (Fig. 19). The consortium consisted of *Chlorella sorokiniana*, identified through phylogenetic analysis of products from PCR targeting the 18S gene, and *Scenedesmus sp.* based on image analysis. The algae were cultivated in a synthetic media (MWC+Se, Guillard and Lorenzen, 1972), which nutrient content was modified according to the experimental needs. As example, nitrate concentration was manipulated to design an experiment whereby parameters related to the nitrate uptake and storage could be identifiable.

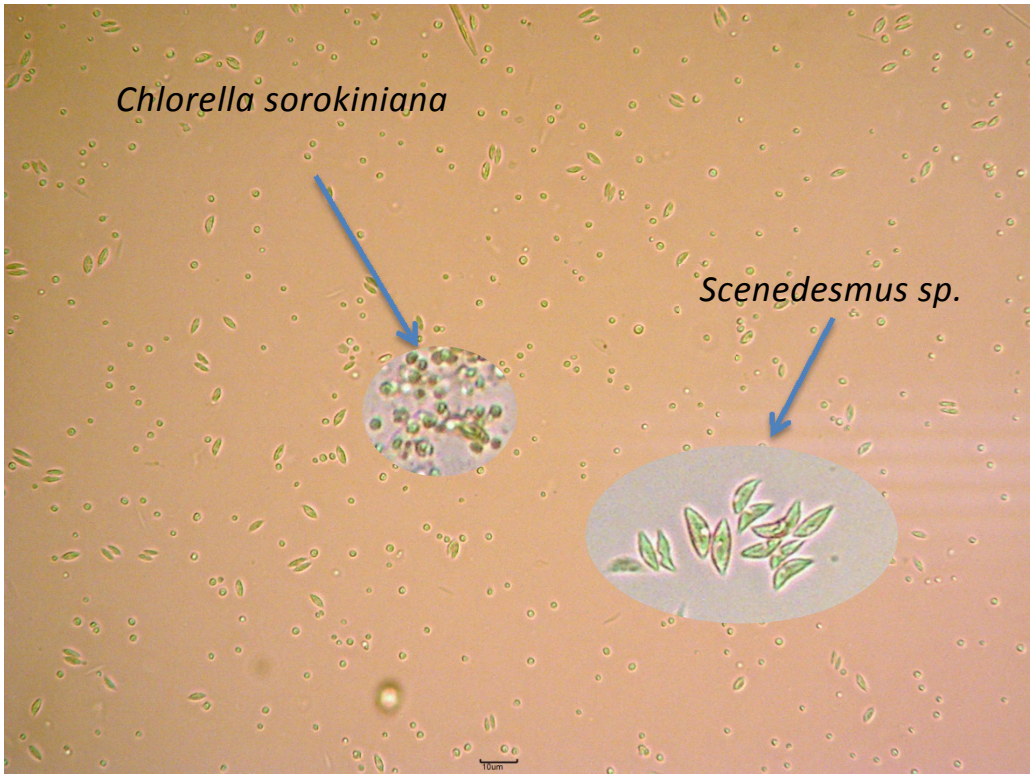

Figure 19: Green microalgae culture used to calibrate the ASM-A (**paper IV**). Picture taken by Michael Steidl.

Experiments were carried out in different laboratory-scale reactors. Micro-batch experiments consisted of 2 ml batches used to infer data for light model calibration (Van Wagenen et al., 2014). Additionally, 1-L batch reactors (Fig. 20) were used to obtain data to calibrate nutrient uptake and storage process model. To ensure parameter identifiability one nutrient at a time was limiting algal growth. Consequently, three different experiments were run to infer the

impact of ammonia, nitrate and phosphate limitation. Out of these two first experiments a first parameter set could be derived and properly identified.

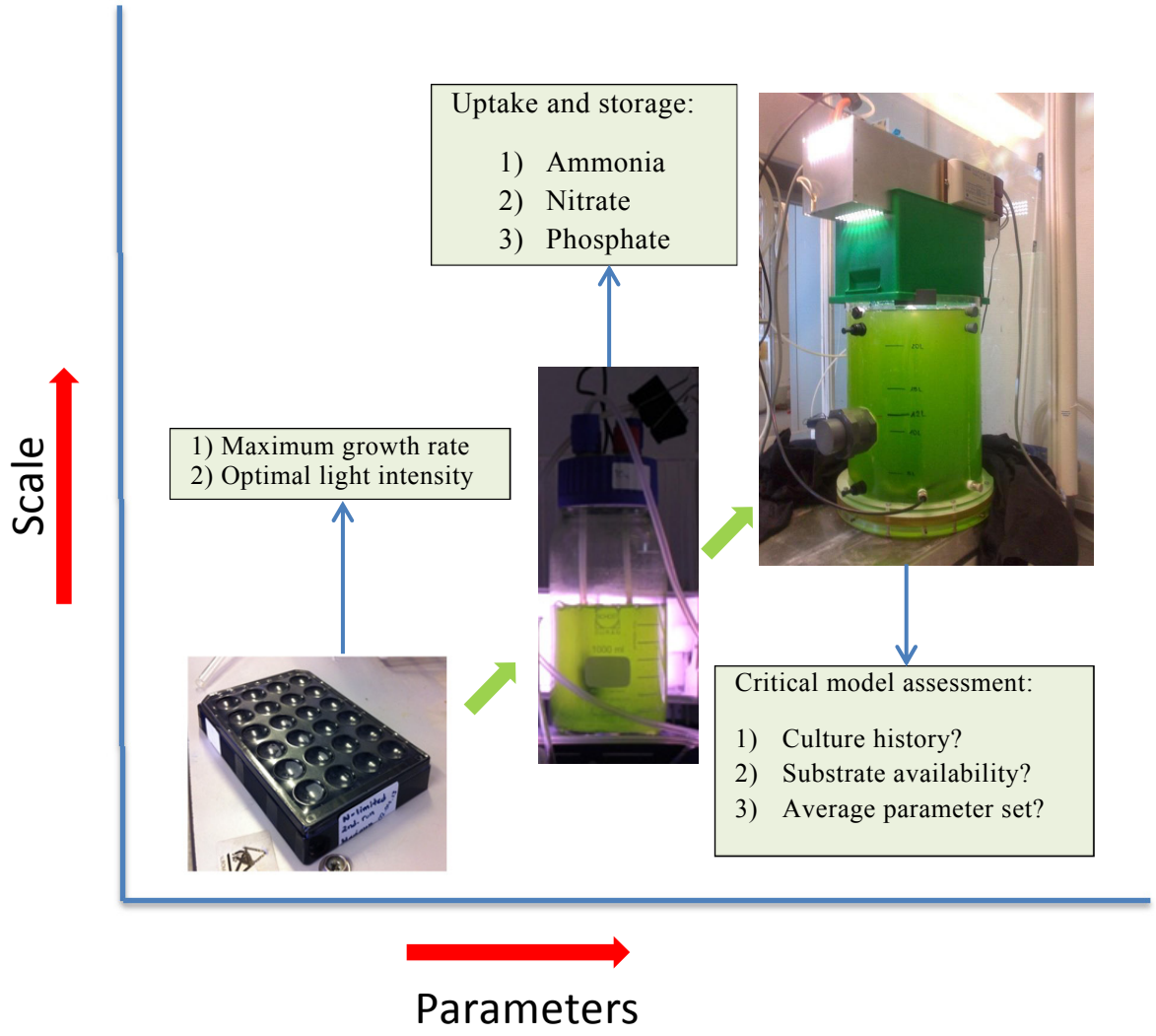

Figure 20: Scheme summarizing the experimental set up and experiment output.

Furthermore, a two-step model evaluation was carried out using data obtained in a 24-L sequenced batch PBR. Through the first evaluation step we aimed to assess the impact of culture history on the estimated parameter values, whilst through the second step it was checked if one could use an average parameter set to reasonable predict the PBR performance. To this end, the substrate availability influence on the parameter values was analyzed. The experiment designed with different initial substrate-to-biomass ratio in each cycle allows distinguishing the effects of culture history from the substrate availability impact (Fig. 21). Through the first evaluation step, parameter sets obtained through each descending cycle were confronted with data obtained

in the corresponding (same initial substrate concentrations) ascending cycle. To evaluate the model accuracy, we used the Janus coefficient, as suggested by Sin et al (2007). In the second evaluation step, Monte Carlo simulations were performed to obtain confidence interval of model predictions (Sin et al., 2009). The uncertainty range for each parameter of the ASM-A was defined as the standard deviation for the parameters estimated in the descending cycles. If the experimental data fall within the confidence interval we considered that the impact of substrate availability was non important.

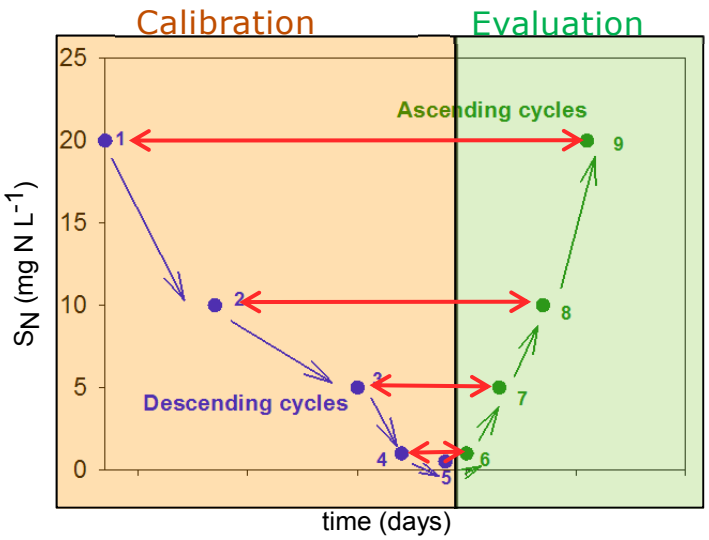

Figure 21. Schematic representation of the experimental design to assess the impact of substrate availability and culture history.

The outcome of the first evaluation step indicates that, for those parameters sensitive to algal biomass, ammonia and phosphate concentrations as well as the nitrogen and phosphorus storage, the source of parameter variability is not related to culture history (J~1). As for the second evaluation step, most of experimental values are in the proximity of the simulation results obtained using mean parameter values (Fig. 22a, 22b, 22d and 22f). Therefore, we suggest that practitioners can calibrate ASM-A using the mean parameter values with their associated uncertainty from **paper IV** as default to model mixed cultures where *Chlorella sp.* and *Scenedesmus sp.* are the dominating species. The discrepancy between measured and simulated data can be explained by parameter variability (i.e. data falls within the confidence interval).

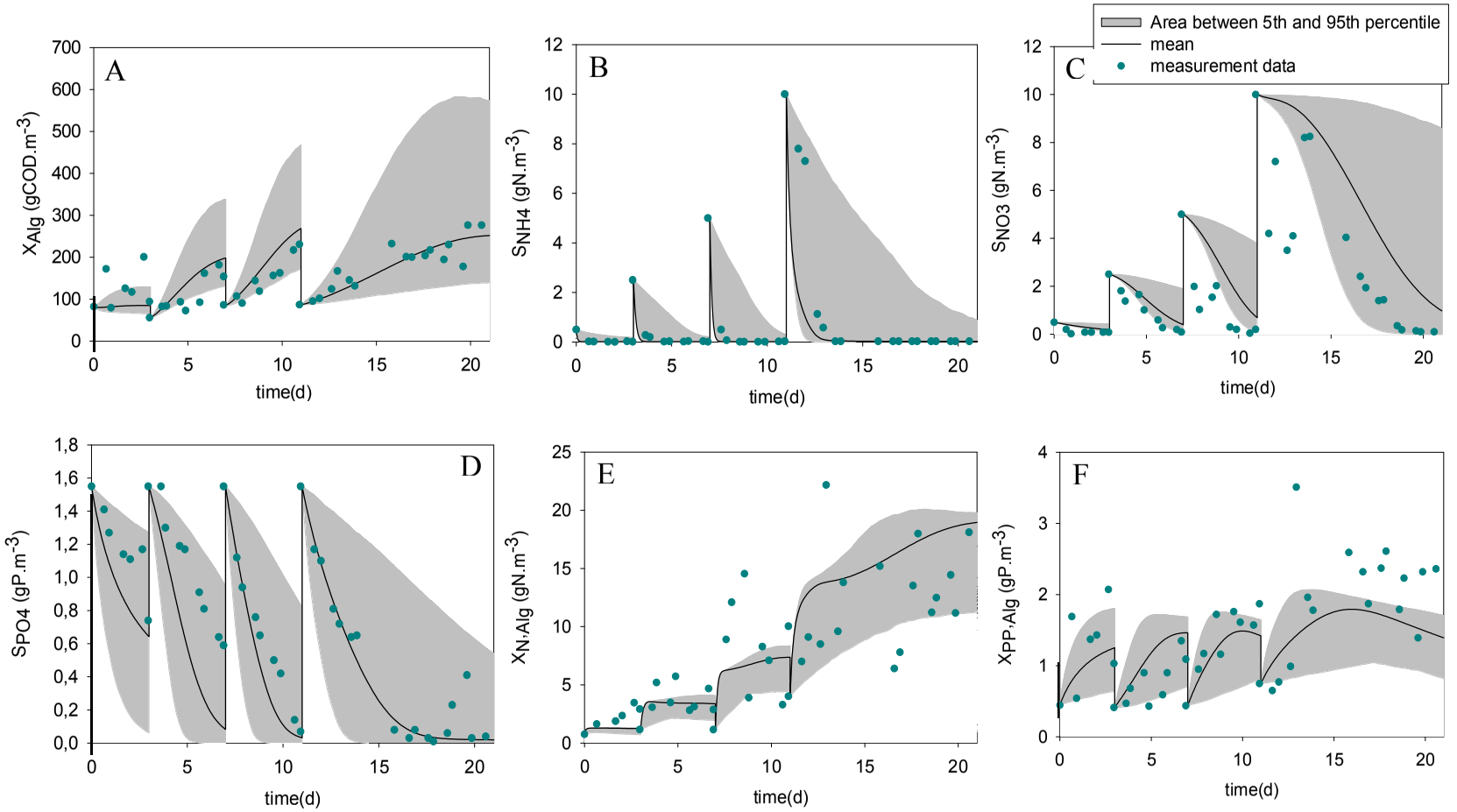

Figure 22. Model evaluation in terms of A) algal biomass, B) ammonia concentration, C) nitrate concentration, D) phosphate concentration, E) internal nitrogen storage, and F) internal phosphorus storage (**paper IV**).

Nitrogen storage can be predicted using the estimated parameters from the descending cycle to assess the model fitting in the parallel ascending cycle (i.e. J~1). In the second step, however, the discrepancy between predicted and measured nitrogen storage cannot be explained through parameter variability (i.e. most data falls outside the predictive confidence interval, Fig 22e). Therefore, the source of parameter variability is substrate availability. Finally, the bulk nitrate concentration prediction fails for both steps (J>>1 and experimental data falls outside the predictive confidence interval, Fig. 22c). This suggests that values of the parameters affecting this model output depend on the history of the culture. According to the GSA the most sensitive model parameter affecting the soluble nitrate concentration is the maximum uptake rate of nitrate (k_{NO}). Since k_{NO} is identifiable, case specific calibration of k_{NO} is proposed. Results suggest there is hysteresis, as the k_{NO} value depends on the previous state of the algae: feast and famine (Fig. 23).

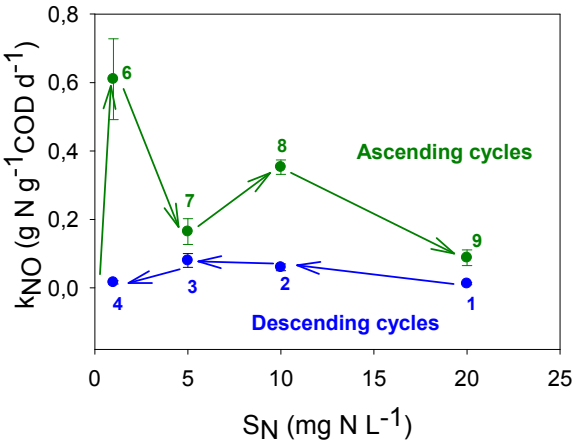

Figure 23. Values of k_{NO} for each of the cycles illustrating the impact of the culture history (**paper IV**).

Taken together, the calibration of ASM-A is not expected to be significantly influenced by parameter variability caused by culture history and substrate availability if proper experimental design is carried out. One exception is the nitrate-nitrogen uptake and storage process, which depends on culture history and substrate availability.

4 Environmental impact assessment of the TRENS system in the Copenhagen area

Finally, the environmental impact of the TRENS system implemented to fulfill the fertigation requirements in the Copenhagen area was analyzed. Life cycle assessment (LCA) has been proved to be a suitable tool to analyze the environmental impact of WWTPs (Corominas et al., 2013) and also useful tool to guide process design towards sustainable solutions (van Boxtel et al., 2015). Therefore, we used LCA to highlight the bottlenecks of the TRENS system, to find potential solutions to overcome them and to identify areas that should be subject of further research.

The TRENS system was implemented as a side-stream process treating 10% of the influent wastewater to Lynetten WWTP, based on reported water demand by local farmers. The chosen crop was winter wheat, which demands a fertilizer with an N-to-P ratio of 16 (Olesen et al., 2009), close to the ratio of the algae used in our studies. The model from **paper I** was adapted to model the side-stream TRENS system and used to provide the data needed for the life cycle inventory (LCI).

TRENS reduced the environmental impact of the WWTP by offsetting the mineral fertilizer production and by reducing the flow treated by the conventional EBPR process. The fraction of wastewater treated by the EBP2R does not undergo nitrification-denitrification processes, which are expected to contribute to N_2O emissions (Kampschreur et al., 2009). Sensitivity analysis identified N_2O emissions as a priority parameter that will need to be subject of further research. Importantly, we have assumed that the PBR does not contribute to N_2O emissions, which contradicts the study by Guiyesse et al. (2013). Therefore, further research should focus on the characterization of N_2O emissions from the PBR. There is a decrease in marine eutrophication due to the avoided discharge of nitrate-nitrogen to the sea, which is stored in algae instead. However, the run-off of the nutrients from the algae may contribute to eutriphication as well. Therefore, future studies should focus on the use of green microalgae as natural fertilizer and nutrient mobility.

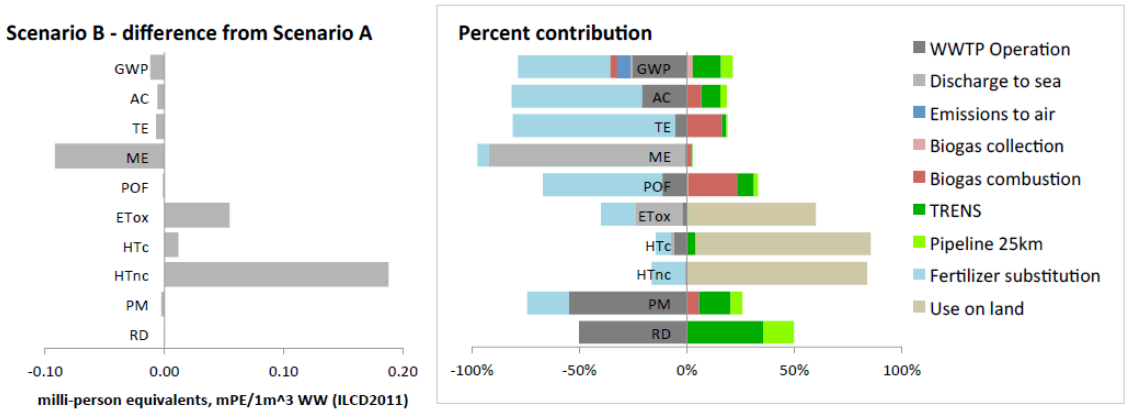

Figure 24. Environmental performance of fertigation with TRENS relative to baseline scenario (**paper V**).

On the negative side, there is a considerably increase in the human toxicity non-cancer effects category (HTnc). This is a consequence of the application of the algal biomass on land as well as irrigation with the treated effluent, which contains heavy metals. Future research should address the fate of the heavy metals in the TRENS system. Changes on the environmental performance are illustrated in Fig. 24.

5 Conclusions

This PhD project presents a novel biological resource recovery scheme that consists of two stages. The upstream process is an enhanced biological phosphorus removal and recovery (EBP2R) activated sludge process. The downstream process is a PBR used for green microalgae cultivation. This combined bacterial-algal system is additionally combined with anaerobic digestion for energy recovery and completely autotrophic nitrogen removal to remove the non-recoverable nitrogen.

The main findings of this work are:

- In this thesis the EBP2R process has been demonstrated to be effective to separate and recover phosphorus and nitrogen from municipal wastewater via model-based studies.
- Results obtained using model-based process development were used to identify the main process parameters that can be used to meet the requirements for optimal green micro-algal growth cultivation for a comparably wide range of algal species. Some of the main process parameters identified comprise the SRT (set to a low level compared to conventional EBPR systems) and the P-stream diversion flow rate.
- A model-based assessment of EBP2R implemented was used to assess the impact of the process configuration, i.e. sequencing batch and continuous-flow system, on phosphorus recovery. Results obtained demonstrate that the sequencing EBP2R allows higher phosphorus recovery via P-stream diversion, recovering maximum 72% of the influent phosphorus. In comparison, the continuous flow EBP2R can recover about 65% of the influent phosphorus.
- The phosphorus recovery by the EBP2R can be limited by the system SRT and the nitrate presence in the anaerobic phase or reactors, thereby leading to anoxic environments that favor denitrification over phosphorus release. The EBP2R process performance in continuous flow can also be deteriorated by the PAOs growth limitation by phosphorus availability in the aerobic reactors. Furthermore, the diversion of the P-stream differently affects the anaerobic and aerobic SRTs, because increasing Q_P results in increased activated sludge concentration in the aerobic tank.
- A control system has been developed for the EBP2R implemented in sequencing batch and the continuous flow process configurations. Using dy-

namic input disturbance scenarios, the sequencing batch shows less variable effluent N-to-P ratio than the continuous system. The control system for the continuous flow configuration fails to meet the operational objectives when the influent nitrogen or phosphorus become limiting.

- The EBP2R process was implemented in laboratory-scale both as CFS and as SBR configurations treating municipal wastewater supplemented with propionate. Experimental data and molecular analysis data obtained with the EBP2R systems employing zero P-stream diversion show that both setups can be prone to extreme filamentous bulking caused mainly by *Thiothrix sp*. bacteria. *Thiothrix* growth is found to be promoted by reduced sulphur compounds, such as sulphide, produced by SRBs. Different operational strategies were tested, and filamentous bulking was successfully mitigated for mainly by manipulating the anaerobic SRT.
- During the filamentous bulking event phosphorus removal was found to be deteriorated by the SRBs activity. SRBs are hypothesized to interact with PAOs by competing for VFAs or by inhibiting them producing sulphide under anaerobic conditions. Phosphorus removal could be successfully restored in the sequencing batch reactor, but not in the continuous-flow system.
- In addition to the activated sludge system, green micro-algal growth on wastewater resources was studied. A set of differently scaled laboratory experiments were designed to infer data suitable for identifying and evaluating a process model for micro-algal growth and nutrient storage. The green-microalgae model was developed in the activated sludge framework.
- An independent experimental data set was inferred and used for evaluating the micro-algal model. Importantly, the experimental design allowed for distinguishing between the impact of the culture history and the impact of substrate availability on the model parameters estimates. Results obtained demonstrate that the maximum nitrate uptake rate depends on the culture history, being higher after starvation periods.
- The models developed in this study were employed in a LCA study, aiming at identifying the strengths and weaknesses of the TRENS system used for resource recovery via fertigation. LCA results highlight that the environmental benefits of nutrient recycling through fertigation may be countered by the discharge of heavy metals on land. Furthermore, the LCA suggests focusing future research efforts on investigating the use of green microalgae as natural fertilizer and N_2O emissions from algal cultivation.

6 Future perspectives

The work presented in the thesis demonstrated the feasibility of the EBP2R system using the ASM-2d. However, the experimental work carried out indicates that the process optimization and assessment carried out in the **papers I** and **II** led to model falsification (Sin et al., 2006). The optimal operational conditions induced changes in the microbial populations that the ASM-2d does not account for and thus cannot predict, e.g., the proliferation of filamentous bacteria and its impact on the abundance of PAOs in the system. The experimental work demonstrates that model falsification may be a general issue for low SRT EBPR systems. Therefore, future research should address the improvement of the existing models to capture the microbial dynamics found in **paper III**. More specifically, the model should include the interactions of SRBs with other bacteria relevant to EBPR functionality, like *Thiothrix* or PAOs. There are already some models available describing SRBs dynamics in anaerobic digestion processes which could be used to extend the available benchmark models (Jeppsson et al., 2013). Given the importance of filamentous bacteria, we suggest making an effort on reducing the uncertainty on the settling model parameters by establishing relations between them and the abundance of relevant filamentous bacteria (e.g. Wágner et al., 2015b).

Future research should also give a proof of concept of the EBP2R system in both laboratory and pilot scale. Based on the findings from this thesis, both based on simulation and experimental studies, the sequencing batch configuration of the EBP2R process is the most effective option. The sequencing batch EBP2R has been demonstrated to be less prone to filamentous bulking and has the potential to recover more phosphorus than the continuous flow EBP2R in the same scenario. Therefore, it is suggested focusing research efforts on the development of the sequencing batch EBP2R.

The control studies show some limitations in keeping a stable N-to-P ratio in the effluent under dynamic conditions. While the deviation from optimal N-to-P ratio is not severe, it would be important to study the effluent quality of a PBR subjected to similar N-to-P variations in the influent. Another important aspect is culture stability. The N-to-P ratio is a valuable tool to drive competition between algal species in algae consortia (Beuckles et al., 2015). Ongoing research focuses on the elucidation of the impact of the N-to-P ratio dynamics on the culture stability of pure and mixed cultures grown in treated municipal wastewater (Steidl, 2015). The control structures could be improved by including the PBR in the control scheme. Control systems for PBR

could benefit from available sensors to monitor conventional WWTPs. Therefore, ongoing research focuses on the development of protocols to calibrate UV-Vis probes to monitor nitrate, biomass or pigments in PBRs (Steidl 2015).

Energy recovery is not explicitly addressed in this work. However, parallel research within this project framework evaluates the energy recovery through anaerobic digestion of green microalgae, the activated sludge from the short SRT EBPR system and the combination of both biomasses. As part of this study, the bioflocculation of algal biomass with activated sludge is also explored (Wágner et al., 2015a).

In this work a green microalgae model is proposed, describing the biokinetics of algal growth and nutrient storage. In the future, it is expected that model predictions can be improved by including a proper physicochemical model extension. The factors that should be included are an improved light model, which considers algal pigmentation and biomass concentration, gas transfer, precipitation processes and pH.

Finally, the presented work aims to describe optimal solutions to recover resources. However, the real pollutants should not be overlooked. The LCA study already highlighted the detrimental impact of heavy metals, which are disposed on land together with the recovered resources. Future research should address the fate of heavy metals in combined bacterial-algal systems, focusing on the impact of low SRT on the removal efficiency by the EBP2R (Sterritt and Lester, 1981). Special attention should be paid to the fate and removal of heavy metals at the nano-scale, which has been recently recognized as knowledge gap (Kirkegaard et al., 2015).

Micropollutants, such as pharmaceuticals or personal care products (PPCPs), should be also removed to ensure safe resource recovery. Legislation is moving towards the recognition of these substances as harmful to the environment (Plósz et al., 2013). Therefore, the recycling of PPCPs together with the different resources should be minimized. This is of special concern for novel resource recovery technologies relying on low SRT systems, such as the EBP2R system. The critical SRT to enhance PPCP removal in conventional activated sludge systems ranges between 7-20 days (Clara et al., 2005; Plósz et al., 2012), which is significantly higher than the desired SRTs for energy recovery. Furthermore, the avoidance of nitrification and denitrification processes, reported to enhance the mineralization of several micropollutants (e.g. Suarez et al., 2010; Fernández-Fontaina et al., 2012), may have a detrimental

effect on the removal efficiency. LCA studies could also include the environmental impact of PPCPs (see e.g. Alfonsín et al., 2014) to assess the most sustainable solutions for their removal.

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In this online version of the thesis, **paper I-IV** are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from.

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The Department of Environmental Engineering (DTU Environment) conducts science-based engineering research within four sections:
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